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Dated: May 26, 2009 Signature: /SharonM. Sintich Reg. No. 48,484/
(Sharon M. Sintich)

Docket No.: 9189
(01017/40451B)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Manfred Brockhaus et al.

Application No.: 08/444,790

Filed: May 19, 1995

For: HUMAN TNF RECEPTOR

Confirmation No.: 5612

Art Unit: 1646

Examiner: Zachary Howard

REPLY BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

This Reply Brief is submitted in response to the Examiner's Answer mailed in this application on March 24, 2009. This Reply Brief is timely filed and no fees are believed due. Any additional required fee may be charged, or any overpayment credited, to Deposit Account No. 13-2855.

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REGULATIONS

I. STATUS OF CLAIMS

1. Claims canceled: 1-61, 63-101, 104, 108, 109, 112, 115-118, 122 and 138

2. Claims withdrawn from consideration but not canceled: 139

3. Claims pending: 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 123-137 and 140-144

4. Claims allowed: none

5. Claims rejected: 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 123-137 and 140-144

6. Claims on appeal: 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 123-137 and 140-144

II. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. For the purpose of responding to the written description rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, and 123-137 under 35 U.S.C. § 112, first paragraph, claims are grouped as follows:

1. Claims 62, 102, 103, 105, 107, 110, 111, 113, 114, 119, 120, 123, 124, 129, 130, 132, 133, and 137

2. Claims 106, 125, 126, and 128

3. Claims 121, 131, 134, and 136

4. Claim 127

5. Claims 103, 107, 111, and 128

6. Claim 135

B. For the purpose of responding to the obviousness rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 125-131 and 134-137 under 35 U.S.C. § 103(a), over Dembic *et al.*, *Cytokine* 2:231-237, 1990 ("Dembic") in view of Capon, U.S. Patent No. 5,116,964 ("Capon"), claims are grouped as follows:

1. Claims 62, 102, 103, 107, 110, 111, 119, 120, 129, and 130

2. Claims 105 and 113

3. Claims 106, 125, 126 and 128

4. Claim 114

5. Claim 121

6. Claim 127

7. Claims 131 and 134-136

8. Claim 137

C. For the purpose of responding to the rejections of claims 140-144 under 35 U.S.C. § 112, first paragraph, claims are grouped as follows:

1. The new matter rejection of claims 140-144

III. ARGUMENT

Summary of Argument in Reply

a. The written description rejection

The Answer has clarified a number of issues regarding the written description rejection of the claims directed to fusion proteins, which comprise (1) a TNF-binding soluble fragment of the 75 kD human TNF receptor (p75 TNF receptor), and (2) a fragment of an immunoglobulin heavy chain constant domain. At page 4, the Answer confirms that there is no dispute regarding written description of the full scope of the immunoglobulin-derived portion of the fusion protein. The written description issue before the Board relates to the TNF receptor soluble fragment portion of the fusion protein. There is also no dispute with respect to the meaning of "soluble fragment", which is acknowledged to refer to the extracellular domain of the TNF receptor or to fragments of this extracellular domain, consistent with usage of the term in the art at the time of filing. Page 34 of Answer (1st full paragraph).

The Answer admits that Appellants had actually isolated and reduced to practice a full length insoluble p75 TNF receptor protein. Pages 24 (3rd paragraph) and 28 (2nd full paragraph) of Answer. The Answer also acknowledges that the prior art (Smith (1990)[1] and Dembic[2]) taught the full length sequence of p75 TNF receptor, and the extracellular domain of the receptor. Pages 11-12 of Answer. The Answer further confirms that, as of the effective filing date, the skilled artisan would have expected this extracellular domain to bind TNF. Page 34 (1st full paragraph) of Answer.

Finally, the Answer agrees that the physical characteristics of the p75 TNF receptor recited in the claims (*i.e.*, a specified molecular weight and N-terminal 18-amino-acid sequence of SEQ ID NO: 10) would uniquely identify the human p75 TNF receptor. Page 49 (2nd full paragraph) and page 50 (3rd full paragraph) of Answer. SEQ ID NO: 10 is acknowledged to correspond to amino acids 1-18 of the Smith (1990) and Dembic sequences. Page 12 of Answer.

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[1] Smith *et al.*, *Science* 248: 1019-1023, 1990; denoted herein as "Smith (1990)" [Appendix B-211].
[2] Dembic *et al. Cytokine* 4: 231-237, 1990, denoted herein as "Dembic" [Appendix B- 80]

The remaining written description rejection has two aspects. One aspect is the Examiner's assertion that the specification does not disclose TNF-binding soluble fragments of p75 TNF receptor because Fig. 4 does not provide the full length sequence of this receptor. Fig. 4 displays a sequence corresponding to amino acids 49-439 of the 439-amino-acid receptor. The Examiner alleges, *inter alia,* that the specification "teaches away" from using the full length extracellular domain because the soluble fragments contemplated are limited to fragments of Fig. 4. This allegation ignores the undisputed facts that:

(1) Appellants actually reduced to practice a p75 TNF receptor protein containing the full length extracellular domain (Examples 1-7 at pages 20-34 of the Specification),

(2) Fig. 4 is explicitly described as a "partial" (*i.e.*, incomplete) sequence throughout the specification (at page 10, lines 23-26; page 10 lines 28-29; and page 35, lines 22-23),

(3) the specification states that "soluble TNF-binding protein fragments [are] cut out from the *complete* sequence" of receptors (page 14, lines 32-36 of the Specification, emphasis added),

(4) the specification describes TNF-binding proteins as "containing" the amino acid sequence of Fig. 4 or fragments thereof, *i.e.* as including proteins that contain *more* than the sequence of Fig. 4 (at page 3, lines 25-27; page 5, lines 11-14; page 7, lines 13-25),

(5) the specification at page 10, line 10 cites to prior art, Smith (1990) [Appendix B-211], which disclose the full length p75 TNF receptor sequence and the extracellular domain of the receptor (pages 11-12 of Answer),

(6) the prior art publication Dembic [Appendix B-80], co-authored by five of the six inventors of the instant application, also discloses the full length p75 TNF receptor sequence and the extracellular domain of the receptor (pages 11-12 of Answer),

(7) page 39 of the Answer admits that "the Lyman Declaration provides evidence that at the time of filing one of skill in the art would look to publications in the prior art to complete the sequences of partial cDNAs," and

(8) in the examples, which are described as illustrative, Appellants fused the *full length* extracellular domain of p55 TNF receptor to all of the domains of the constant region of a human IgG heavy chain other than the first domain (Example 11 at pages 42-43 of the Specification).

The admitted fact that Dembic disclosed the full length p75 TNF receptor sequence is evidence that the inventors possessed the full length sequence before the application's priority date. It defies logic and is contrary to the teaching of the specification to assert that Appellants, despite possessing the purified full-length protein and knowing its sequence, and despite providing a citation to the Smith (1990) publication which disclosed the full-length sequence, intentionally limited their invention to fragments of the partial sequence displayed in Fig. 4.

The other aspect of the written description rejection relates to support for the scope of the claims. Both the scope of the claims and the written descriptive support for this scope are disputed. The Examiner asserts that a "soluble fragment" of "human TNF receptor" of about 75 kD includes mutated variants of the naturally occurring receptor. This assertion is unreasonable and inconsistent with the specification's use of the terms "human" and "receptor." Nothing in the specification would indicate that "human TNF receptor" encompasses mutated variants. The specification uses the term "receptor" to refer to specific naturally occurring receptors and never uses this term to refer to fragments or analogues. See, *e.g.*, page 2, lines 1-4, page 7, lines 13-18, page 14, lines 32-36 and page 35, lines 11-18 of the Specification. Instead, the specification describes fragments and analogues using the term "TNF binding protein." The Examiner's claim construction is impermissible because it would render the term "human" meaningless. The Examiner's position is also inconsistent with the general contemporaneous use of "human" in the art and, moreover, contrary to factually parallel Federal Circuit case law holding that "human" excludes mutated variants.

The Examiner's further assertion that the scope of the claims extends to fragments as small as a single amino acid has been disproven by Appellants' unrebutted evidence that such small fragments would not have bound TNF. The Answer asserts at page

46 that residues 10-54 at the N-terminus of p75 TNF receptor are required for TNF-binding activity. Appellants provided evidence that shortening the C-terminus of p75 TNF receptor from residue 162 to residue 142 destroys TNF-binding activity.[3] The Examiner's refusal to consider this evidence is legal error. Controlling precedent has approved the use of later publications as evidence of facts regarding the state of art existing on the filing date of an application. *See In re Hogan*, 559 F.2d 595, 605, 194 U.S.P.Q. 527, 537 (C.C.P.A. 1977).

Thus, the scope of the claims does not extend to an exponential number of mutated variants. Nor do the claims encompass fragments as small as one amino acid. Properly construed, the claims encompass a limited group of fragments of an amino acid sequence that is already fully known. Based on the observations described above, a soluble TNF-binding fragment would need to include some portion of amino acids 10-162, and must include at least amino acids 54-142. In this context, Appellants' representative species, depicted in the diagram below at page 19 (section III.A.4), are broadly distributed throughout the scope of the claim and provide sufficient written descriptive support. Independently, the knowledge in the prior art regarding the location of the TNF-binding region at amino acids 17-178 provides a structure-function correlation adequate for written descriptive support. Together, the representative species and knowledge of structure-function relationships are ample support for the scope of the claims.

Moreover, Appellants' written descriptive support is even more compelling for each of claims 103, 107, 111, 128 and 135. Each of these claims requires that the soluble fragment include SEQ ID NO: 10, which corresponds to the N-terminal 18 amino acids of the p75 TNF receptor. Thus, these claims recite an even more limited group of TNF-binding soluble fragments because at least amino acids 1-142 must be present.

All of the claims argued separately in the Appeal Brief should be considered separately for the reasons set forth in the Appeal Brief.

b. The obviousness rejection

The Answer has also clarified issues regarding the obviousness rejection. The acknowledged unexpected results show that the combination of the two elements in the fusion protein, *i.e.* the TNF receptor portion, and the immunoglobulin portion, is not carrying

[3] See U.S. Patent No. 5,395,760; col. 22 lines 5-49 [Appendix B-219]

out the function that would be predicted for each element separately. The Examiner agreed that the drastic reduction in two different types of effector function (pages 62), the failure to form aggregating complexes (page 63), the increase in TNF neutralizing potency (page 64), the increase in kinetic stability (page 65) and the improvement in TNF inhibition (page 65) were each unexpected results. Such unexpected results mandate a finding of nonobviousness in view of the Supreme Court's decision in *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 82 U.S.P.Q.2d. 1385 (2007).

The Answer admits that "the immunoglobulin heavy chain constant region has pro-inflammatory activity that is retained by fusion proteins of such, or that soluble fragments of TNFR could act as an anti-inflammatory agent." Page 57 (2nd paragraph) of Answer. Thus, the art teaches away from combining such a pro-inflammatory immunoglobulin fragment disclosed in Capon[4] with the anti-inflammatory TNF receptor fragment disclosed in Dembic.

However, the Examiner relies on an asserted *in vitro* use of the claimed fusion protein for affinity purification of TNF as motivation to one of skill in the art to combine the two elements. This asserted use still fails to supply an adequate rationale for selecting, in particular, fusion proteins that comprise the hinge, CH2 and CH3 immunoglobulin domains, from among the hundreds or even thousands of possible variations of fusion proteins contemplated. The important functioning element for affinity purification of TNF would be the presence of the TNF-binding fragment of TNF receptor, and the endless possibilities for added sequence might include (1) no added sequence at all, (2) any portion of a non-immunoglobulin peptide, (3) any portion of an immunoglobulin, or (4) any portion of the constant region of an immunoglobulin, *e.g.,* CH1 alone, hinge alone, CH2 alone, CH3 alone, a fragment of any of these domains, or some combination thereof.

Moreover, the acknowledged evidence of unexpected and advantageous results rebuts any alleged *prima facie* case of obviousness. The Answer admits that Appellants' evidence demonstrates six different types of unexpected properties for two different fusion proteins comprising a p75 TNF receptor soluble fragment fused to all the domains of a human IgG heavy chain constant region other than CH1.

[4] U.S. Patent No. 5,116,964 denoted herein as "Capon" [Appendix B-26]

Finally, the asserted motivation to make the claimed fusion protein for use in affinity purification of TNF completely fails to apply to dependent claims 114 and 137. These claims recite a "pharmaceutical composition" comprising a "pharmaceutically acceptable carrier material." Non-sterile and possibly toxic or contaminated solutions are not pharmaceutically acceptable carrier materials. Similarly, affinity purification compositions are not "pharmaceutical compositions."

The Examiner had previously stated that sterile and isotonic solutions meet the definition of a "pharmaceutically acceptable carrier" as recited in claims 114 and 137. See page 15 of Final Action. The Examiner asserted that one was motivated to resuspend the claimed fusion protein, assertedly made for use in purifying TNF by affinity, in the sterile and isotonic formulations described in Capon, without any factual basis or logical scientific reasoning to support the allegation that affinity purification requires sterile and isotonic formulations. See page 15 (last two sentences) and top of page 16 of Final Action.

The Examiner now makes an "about face," and asserts that "pharmaceutical compositions" of proteins and "pharmaceutically acceptable carrier materials" encompass non-sterile water and possibly toxic or contaminated solutions for use in affinity purification. Affinity purification involves cross-linking by chemical agents, including derivatization with bifunctional agents as described in Capon. The Examiner's assertion that an injectable pharmaceutical composition comprising an immunosuppressive protein need not be sterile is wrong. Sterility of compositions comprising a therapeutic protein is mandated by law (*see, e.g.,* 21 C.F.R. § 610.12), and evidenced by statements in the art of record.

The Examiner has impermissibly ignored the "pharmaceutical composition" limitation in the preamble of these claims. A claim's preamble must be read as a meaningful limitation when it results in a physical difference in the product claimed, and particularly when it distinguishes the prior art. See *In re Stencel*, 828 F. 2d 751, 4 U.S.P.Q.2d 1071 (Fed. Cir. 1987); *Catalina Mktg. Int'l, Inc. v. Coolsavings.com, Inc.*, 289 F.3d 801, 808-809, 62 U.S.P.Q.2d. 1781, 1787 (Fed. Cir. 2002). In the instant case, the pharmaceutical use recited in the preamble limits the physical characteristics of the claimed composition in a way that excludes non-sterile or contaminated solutions destined for use in affinity purification.

The Examiner has also impermissibly ignored the limitation "pharmaceutically acceptable carrier materials." The art of record, including Capon, teaches that sterile and

isotonic formulations are pharmaceutically acceptable carrier materials. In this case, the limitation "pharmaceutically acceptable carrier materials" also excludes non-sterile or contaminated solutions destined for use in affinity purification.

All of the claims argued separately in the Appeal Brief should be considered separately for the reasons set forth in the Appeal Brief.

c. The new matter rejection

The Answer withdrew the enablement rejection of claim 140-144 but maintained the new matter rejection. Page 22 of Answer.

The new matter rejection is based in part on the pervasive misconception that the specification does not describe cDNA encoding full length p75 TNF receptor simply because Figure 4 does not display the full sequence. This is erroneous for the same reasons outlined above regarding the similar written description rejection.

The Examiner also argues that the deposit reference, to a particular DNA encoding an insoluble TNF binding protein of about 65/75kD, is improperly inserted into the specification because the adjacent phrase describes a genus of such DNAs. This argument fails in view of factually parallel case law which allowed applicants to insert a deposit reference to a particular monoclonal antibody where the specification described a genus of monoclonal antibodies.

A. The written description rejection of claims 62, 102, 103, 105, 107, 110, 111, 113, 114, 119, 120, 123, 124, 129, 130, 132, 133, and 137

1. Full length, TNF-binding human p75 TNF receptor was actually reduced to practice and described in the specification

As noted above, one aspect of the written description rejection is the Examiner's position that all of the specification's disclosure of soluble fragments must be limited to the partial p75 TNF receptor sequence set forth in Fig. 4. However, the

Examiner's assertion is based on a contorted interpretation of the specification and ignores an overwhelming number of undisputed facts that completely contradict his assertion.

The Examiner has also committed legal error by insisting on his personal interpretation of what the specification conveys to the skilled artisan, in the face of contrary factual evidence provided in the Lyman Declaration. *See In re Alton*, 76 F.3d 1168, 1174, 37 U.S.P.Q.2d 1578, 1582-83 (Fed. Cir. 1996). The Lyman Declaration [Appendix B-143] fully rebuts the Examiner's unsupported interpretation of the specification. The evidence in the declaration is self-explanatory and is not reiterated here. The Examiner's failure to provide factual rebuttal evidence to support his position is also clear and reversible error. *See In re Spormann*, 363 F.2d 444, 447, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966).

The Answer acknowledges that Appellants actually reduced to practice an insoluble p75 TNF receptor protein containing the full length extracellular domain. Page 24 of the Answer. Fig. 4 is explicitly described as a "partial," *i.e.* incomplete, sequence of the TNF receptor throughout the specification. For example:

> There are also preferred DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the *partial* cDNA sequences shown in Figure 4 are preferred. Page 10, lines 23-26 of the Specification; emphasis added.
>
> The peptides IIA, IIC, IIE, IIF, IIG and IIH are coded by the *partial* cDNA sequence in FIG. 4, Page 10, lines 28-29 of the Specification; emphasis added.
>
> Essentially analogous techniques were used to identify 75/65 kD TNF-BP-coding *partial* cDNA sequences . . . Example 8 [referring to Figure 4], at page 35, lines 22-23 of the Specification; emphasis added.

Thus, when Fig. 4 was inserted into the application on April 20, 1990, the specification made clear to others skilled in the art that Fig. 4 is an incomplete sequence. The specification also explicitly stated that soluble fragments of the p55 and p75 TNF receptors were to be cut out from the "complete" sequence:

> On the basis of the thus-determined sequences and of the already known sequences for *certain receptors*, those partial sequences which code for *soluble TNF-BP fragments* can be determined and *cut out from the complete sequence* using known methods [42]. Page 14, lines 32-36 of the Specification, emphasis added.

The plain words of this sentence convey to those skilled in the art that soluble TNF-binding protein fragments of receptors are cut out from the *complete* sequence, not a partial sequence.

Appellants added the description of the presently claimed fusion proteins in their priority application filed August 31, 1990. At that time, instead of replacing Fig. 4 with the complete sequence, the inventors added a citation to Smith (1990). See page 10, line 10 of the Specification. Smith (1990) disclosed the full length p75 TNF receptor sequence and the extracellular domain of this sequence. Smith (1990) also stated the sequence was deposited in a public, searchable database under accession no. M32315. See Fig. 3 and legend of Smith (1990) [Appendix B-211].

The Examiner unreasonably alleges at page 26 of the Answer that "Smith [(1990)], which is cited on page 10 solely in reference to deletions from the sequences of Figure 4." It defies logic to assert that, by adding a reference to full length sequence, the inventors meant to remove full length sequence from the scope of their invention. That senseless interpretation would not be the understanding conveyed to one skilled in the art and is also rebutted by the Lyman Declaration [Appendix B-143]. Page 39 of the Answer admits that "the Lyman Declaration provides evidence that at the time of filing one of skill in the art would look to publications in the prior art to complete the sequences of partial cDNAs."

Thus, the Examiner's allegation that the specification's disclosure of fragments is limited to fragments of Fig. 4 is irrational, when that sequence was explicitly stated to be a partial sequence, and when the specification cited to prior art, Smith (1990), which disclosed the full length p75 TNF receptor sequence. Dembic's disclosure of full length p75 TNF receptor sequence also shows that the inventors, co-authors of Dembic, possessed and published this sequence before the application's effective filing date. As discussed below, controlling Federal Circuit precedent holds that applicants need not reiterate a prior art known sequence.

The Examiner's argument at pages 27 and 31 of the Answer that the specification "teaches away from using the entire extracellular domain of the 75 kD receptor as a species of soluble fragment" is based on the unsupported assumption that no sequences longer than Fig. 4 are contemplated by the specification. This assumption is contradicted by the fact that Appellants actually reduced to practice a p75 TNF receptor protein containing the full length extracellular domain, by the numerous explicit statements in the specification

that Fig. 4 is a partial (incomplete) sequence, by the explicit statement that soluble TNF binding proteins are fragments of "complete" receptor sequence, and by the explicit statements within the specification that fragments of proteins "containing" this sequence are contemplated.

Even the passages cited by the Examiner support Appellants' position. Page 27 of the Answer points as support to passages including the following:

> This invention also comprises TNF-binding proteins *containing* amino acid sequences of Figure 1 or Figure 4, proteins containing fragments of these sequences . . . Page 3, lines 25-27 of the Specification; emphasis added.
>
> The TNF-binding proteins of the present invention include homogenous proteins *containing* the amino acid sequence depicted in FIG. 1 or in FIG. 4, proteins containing fragments of either sequence, . . . Page 5, lines 11-14 of the Specification; emphasis added.

These passages (among others, *e.g.,* page 7, lines 13-25 of the Specification) convey that the TNF binding proteins *contain* sequences of Fig. 4, making it clear that these TNF-binding proteins can include additional sequences beyond Fig. 4. Thus, these passages do *not* support the Examiner's position that TNF-binding proteins must consist only of the Fig. 4 sequence or fragments thereof.

A number of different Federal Circuit cases establish that the written description requirement does not require reiteration of known prior art sequences in the specification. *See, e.g., Falkner v. Inglis,* 448 F.3d 1357, 1368, 79 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2006), *Capon v. Eshhar,* 418 F.3d 1349, 1357, 76 U.S.P.Q.2d 1078, 1084 (Fed. Cir. 2005), *Monsanto Co. v. Scruggs,* 459 F.3d 1328, 1336, 79 U.S.P.Q.2d 1813, 1818-19 (Fed. Cir. 2006). The Examiner's attempt to distinguish *Falkner* because of an allegedly different fact pattern fails because a general legal principle is controlling precedent even in different fact situations. The Answer's citation at pages 38-39 to *Fiers v. Revel,* 984 F.2d 1164, 25 U.S.P.Q.2d 1601 (Fed. Cir. 1993) and *Amgen Inc. v. Chugai Pharmaceutical Co.,* 927 F.2d 1200, 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991) is wholly inappropriate to the present situation, where the p75 TNF receptor protein sequence is known and the physical characteristics recited in the claim uniquely identify the human p75 TNF receptor protein sequence. *See, e.g.*, page 49 (2nd full paragraph) and page 50 (3rd full paragraph) of Answer. Even the cases cited by the Examiner recognized that proteins are "claimable by structure *or*

physical properties [emphasis added]" as long as those properties distinguish and identify what is claimed. *Fiers,* 984 F.2d at 1169.

The instant facts are exactly parallel to *Falkner's* fact pattern. In *Falkner,* there was undisputed testimony that the DNA sequence of the entire poxvirus genome and the locations of "essential" poxvirus genes were known in the art. The Federal Circuit held that applicants could rely on this knowledge in the art for written description of essential poxvirus genes that could be deleted or inactivated to make a poxvirus vaccine. The Answer at page 30 characterizes *Falkner* as holding that "the disclosure of the Inglis '040 application describes, in great detail, how to make a vaccine with one type of virus (herpes), which provides a description for the skilled artisan regarding how to make a vaccine with a second type of virus (poxvirus)." In the present case, the application describes how to make an immunoglobulin fusion protein with the extracellular domain of one type of TNF receptor (p55 TNF receptor), which accordingly should provide a description regarding how to make a corresponding immunoglobulin fusion protein with the extracellular domain of a second type of TNF receptor (p75 TNF receptor).

The Answer admits that in Example 11 the extracellular domain of p55 TNF receptor was used to construct an immunoglobulin fusion protein. Page 31, lines 7-9 of Answer. However, the Examiner argues that there is no teaching that a similar extracellular domain should be used for p75 TNF receptor or for TNF receptors in general, and argues that the specification teaches away from using the entire extracellular domain of p75 TNF receptor as a soluble fragment. Page 31, lines 9-14 of Answer. This argument ignores (1) the explicit statement in the specification at page 20, lines 27-30 that the examples are illustrative, (2) the unrebutted evidence in the Lyman declaration at paragraphs 10-12 and 17 that the description and examples of immunoglobulin fusion proteins apply to soluble fragments of *either p55 or p75* TNF receptor, (3) the instruction in the specification to cut TNF binding proteins out from the *complete* sequence of receptors, and (4) the consistent wording of the Specification referring to fragments as *containing* all or part of the sequence of Figure 4, not consisting only of fragments of the sequence of Figure 4.

For all of the reasons described above and in the Appeal Brief, the specification does describe soluble fragments of full length, TNF-binding human p75 TNF receptor.

2. *A "soluble fragment" of a "human TNF receptor" of about 75 kD does not include mutated variants*

With respect to the TNF receptor fragment portion of the claimed fusion protein, the claims recite a "soluble fragment" of a "human TNF receptor" of about 75 kD molecular weight. The Examiner's assertion that this "soluble fragment" of "human TNF receptor" includes mutated variants of the naturally occurring human receptor is (1) not supported by the portions of the specification cited in the Answer, (2) inconsistent with the specification's use of the terms "receptor" and "human", (3) in conflict with the general contemporaneous use of the term "human" in the art, and (4) contrary to factually parallel Federal Circuit case law. While claims during examination are given their broadest reasonable interpretation consistent with the specification, this interpretation must be *reasonable* and must be *consistent with the specification*. See *In re Buszard*, 504 F.3d 1364, 1366, 84 U.S.P.Q.2d 1749, 1751 (Fed. Cir. 2007); *Merck & Co. v. Teva Pharms. USA, Inc.*, 347 F.3d 1367, 1371, 68 U.S.P.Q.2d 1857, 1860 (Fed. Cir. 2003).

Nothing in the specification defines "human TNF receptor" as encompassing mutated variants. The major thrust of the arguments, at pages 5, 11 and 42-44 of the Answer, is that the specification contemplates analogues and variants of the TNF receptor, which include those containing mutations such as deletions, substitutions and additions to the TNF receptor. However, the cited disclosures do not support the Examiner's position because they all relate to the broader term "TNF binding proteins (TNF-BP)" and are not properly applied to the phrase "human TNF receptor." Statements with respect to TNF-BP are simply not relevant to the meaning of "human TNF receptor."

The specification uses the term "receptor" to refer to the naturally occurring, membrane-bound receptor. The term "receptor" is never used to refer to fragments or analogues. In contrast, when describing fragments or analogues, the specification uses the term "TNF-BP." For example, in the following excerpts, fragments are described using the term "TNF-BP" while the natural full-length protein is described using the term "receptor":

> These biological effects are mediated by TNF via *specific receptors*. According to present knowledge not only TNFα, but also TNFβ bind to the same receptors [21]. Different cell types differ in their number of TNF receptors [22, 23, 24]. . . . The separation of *soluble TNF-BP from human serum or urine* by ion exchange chromatography and gel filtration (molecular

> weight in the region of 50 kD) was described by Olsson et al. [30]. Page 2, lines 1-26 of the Specification; emphasis added.
>
> In more detail, the proteins of the present invention are non-soluble proteins, i.e. for example *membrane proteins or so-called receptors*, and *soluble or non-soluble fragments* thereof, which bind TNF (*TNF-BP*), in homogeneous form, as well as their physiologically compatible salts. Page 7, lines 13-18 of the Specification; emphasis added.
>
> On the basis of the thus-determined sequences and of the already known sequences for *certain receptors*, those partial sequences which code for *soluble TNF-BP fragments* can be determined and cut out from the complete sequence using known methods [42]. Page 14, lines 32-36 of the Specification; emphasis added.

Nothing in the specification defines "human" as encompassing mutated variants. The Answer at page 43 points to only two uses of "human" in the specification that allegedly support the position that "human" refers to mutated versions: "human cell lines" or "human radioiodinated ^{125}I-TNF". The first use of the term "human" is simply not relevant in the context of mutations to the amino acid sequence of a polypeptide. The second use of the term human supports the concept that modified or variant proteins are different from "human" protein. The fact that Applicants refer to the radio-labeled human TNF as "human radioiodinated ^{125}I-TNF" is an indication that it differs from "human TNF" and thus required the additional descriptor "radioiodinated ^{125}I."

Appellants had cited to Smith (1990) [Appendix B-211] and Dembic [Appendix B-80] as evidence that those in the art use the term "human" to refer to the naturally occurring human sequence. Although the Examiner disregarded these citations because they "do not comment on mutated versions," the record contains other generally contemporaneous evidence that concerns mutated proteins. For example, the following references support Appellants' position that mutated variants are not referred to as "human" proteins: Hsu & Chao, *J. Biol. Chem.*, 268:16430-16436 (1993) [Appendix B-468] refers to mutated receptors as "mutant" or "chimeric" receptors throughout, *e.g.,* at pages 16430, 16431, 16435, while the naturally occurring protein is referred to as "human TNF receptor," *e.g.,* at pages 16431 and 16432; Kruse *et al, EMBO J.* 11(9): 3237-44 (1992) [Appendix B-475] refers to mutated versions as "variants" or "mutant proteins," *e.g.*, at pages 3238, 3239, while the naturally occurring protein is referred to as "human" or "wild type", *e.g.* at pages 3238, 3239; and Wilks, *Molec. Aspects Med.* 12: 255-265 (1991) [Appendix B-483] refers to

mutated versions as "mutants," *e.g.* at pages 258, 259, 260, while the naturally occurring protein is referred to as "human differentiation antigen CD4" at page 255.

Federal Circuit case law also supports Appellants' position that the term "human" excludes non-naturally occurring mutations. See *Genentech, Inc. v. Wellcome Foundation*, 29 F.3d 1555, 1564-1565, 31 U.S.P.Q.2d 1161, 1168 (Fed. Cir. 1994). In this case, even though the specification defined tissue plasminogen activator (t-PA) as potentially including mutated variants, the Federal Circuit concluded that "the phrase 'human tissue plasminogen activator' . . . means natural t-PA." *Id* at 1565.

The Examiner's position is also contrary to Federal Circuit case law which instructs that "[a]ll words in a claim must be considered in judging the patentability of that claim against the prior art." *In re Wilson*, 424 F.2d 1382, 1385, 165 U.S.P.Q. 494, 496 (C.C.P.A. 1970). The Examiner's proposed reading of the term "human TNF receptor" as including mutated TNF receptors would render the term "human" meaningless. Such a refusal to consider the term "human" as a meaningful limitation is impermissible under well-established and controlling precedent.

Moreover, the phrase "soluble fragment" cannot be read to include mutated variants. The basis for the assertion that "soluble fragment" includes mutations was the assumption that the soluble fragment is derived from a human TNF receptor that itself encompasses mutations. See, *e.g.*, pages 6 and 11 of the Answer. That underlying assumption is wrong, for the reasons discussed above. In the specification, the term "fragment" is separate and distinct from the term "analogue." The term "analogue" is used to refer to substitutions/replacements of the type that would give rise to mutated variants:

> The TNF-binding proteins of the present invention include homogenous proteins containing the amino acid sequence depicted in Figure 1 or in Figure 4, proteins *containing* ***fragments*** of either sequence, and ***analogues*** of any such proteins . . . An ***analogue*** is a protein in which one or more amino acids of the sequences depicted in Figure 1 or in Figure 4 have had their side-groups *chemically modified in a known manner, or those in which one or more amino acids have been replaced* or deleted, without thereby eliminating TNF-binding ability. Page 5, lines 11-23 of the Specification; emphasis added.

Thus, the Examiner's assertion that the claims encompass mutated variants is factually and legally erroneous. No basis exists in the specification for the interpretation that "soluble fragments" of a "human TNF receptor" of about 75 kD include mutated variants of the naturally occurring receptor. The Examiner's assertion is unreasonable, contradicted by the specification's use of these terms, inconsistent with how the skilled artisan generally understands these terms, and contrary to Federal Circuit case law.

3. A TNF-binding soluble fragment cannot be as small as a single amino acid

The Examiner's assertion that the scope of the claims extends to fragments as small as a single amino acid has been disproven by Appellants' unrebutted evidence that such small fragments would not have bound TNF. For example, shortening the C-terminus of p75 TNF receptor from amino acid 162 to amino acid 142 destroyed TNF-binding activity. See U.S. Patent No. 5,395,760, col. 22, lines 5-49 [Appendix B-219] (fragment consisting of amino acids 1-162 of mature p75 TNF receptor retained TNF binding activity but fragment consisting of amino acids 1-142 did not). This data shows that some portion of amino acids 142-162 is required for TNF-binding activity.

As another example, the Examiner asserted elsewhere in the Answer that amino acids 10-54 at the N-terminus are required for TNF binding. See, *e.g.*, pages 28, 32 and 46 of the Answer, citing Chan *et al.*, *Science*, 288:2351-2354, 2000 ("Chan (2000)") [Appendix B-76]. According to the Examiner, therefore, some portion of amino acids 10-54 is required for TNF-binding activity.

These two observations are depicted in the diagram at page 19 below in section III.A.4. Appellants' evidence and the Examiner's assertion lead to the conclusions that truncating the N-terminus to amino acid 54 eliminates the claimed TNF-binding activity, while shortening the C-terminus to amino acid 142 eliminates the claimed TNF-binding activity. Thus, a TNF-binding soluble fragment must contain at least amino acids 54-142 of p75 TNF receptor. Some portion of amino acids 10-162 is required for TNF-binding activity.

While the Answer does not appear to dispute the factual accuracy of Appellants' evidence, the Answer ignores the evidence because it was not published prior to the filing date. However, Appellants' use of later-published evidence for this purpose is entirely permissible, and the Examiner's refusal to consider it is reversible error. *See In re*

Alton, 76 F.3d 1168, 1174, 37 U.S.P.Q.2d 1578, 1582 (Fed. Cir. 1996) (reversible error to disregard evidence submitted by Applicants). The Court of Customs and Patent Appeals "has approved use of later publications as evidence of the state of art existing on the filing date of an application." *In re Hogan*, 559 F.2d 595, 605, 194 U.S.P.Q. 527, 537 (C.C.P.A. 1977). The court stated:

> The difference may be described as that between the *permissible* application of later knowledge about art-related *facts existing on the filing date* and the *impermissible* application of later knowledge about later art-related facts (here, amorphous polymers) which did *not exist* on the filing date. *Hogan*, 559 F.2d at 605, 194 U.S.P.Q. at 537; emphasis added.

The Examiner agrees that it was a fact existing on the effective filing date that the full length sequence (amino acids 1-439) and extracellular domain (amino acids 1-235) of p75 TNF receptor were known in the prior art. Appellants' later evidence shows that fragments of this known sequence that were as small as a single amino acid *would not have specifically bound TNF*. Thus, Appellants' evidence is the permissible application of later publications about facts existing before the filing date and this evidence cannot be disregarded. The Examiner's reliance on Chan (2000), a post-filing publication, is quite analogous. The Examiner cited Chan (2000) to support his assertion that fragments missing amino acids 10-54 would not bind TNF. The Examiner cannot rely on later publications in this manner and yet ignore Appellants' like evidence.

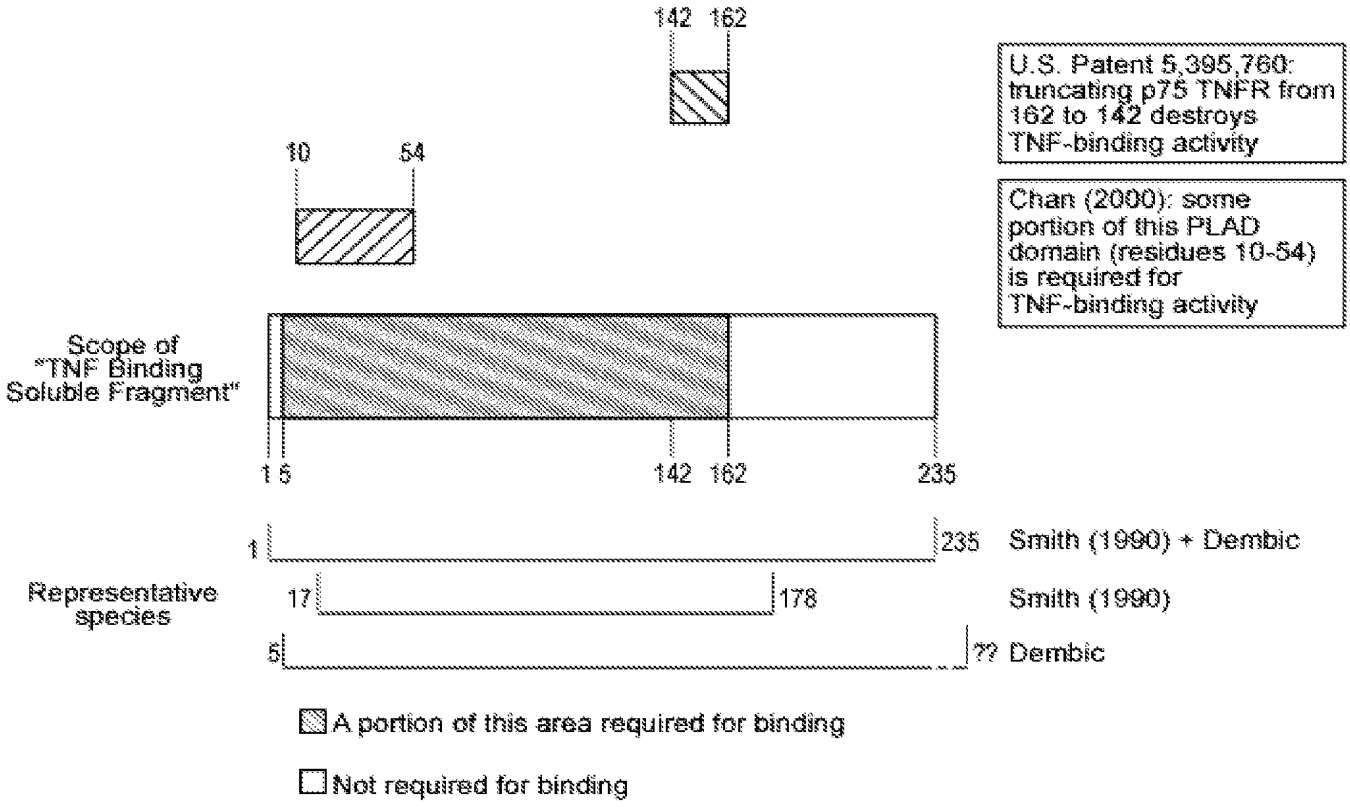
Thus, the Examiner's characterization of the claims as being directed to a "vast genus" of fragments, ranging in size from the entire extracellular domain (amino acids 1-235) to a fragment as small as one amino acid, is clearly erroneous in view of Appellants' evidence and the Examiner's own assertions that such small fragments would not bind.

4. Correction of diagram showing representative species known in the art

Appellants provide herewith a corrected diagram showing disclosures in the prior art with respect to representative species of soluble fragments of human p75 TNF receptor, discussed below in section III.A.5. The diagram corrects an inadvertent error in the Appeal Brief with respect to the disclosure in Smith (1990) that the N-terminal 162 amino acids contain the putative binding site. As the Examiner correctly pointed out, the cited portion of the Smith (1990) reference referred to amino acids 39-200 of Fig. 3. See pages

1020-1021 of Smith (1990) [Appendix B-211]. When this numbering is corrected for the presence of the 22-amino acid signal sequence, the putative binding site referenced in Smith (1990) is amino acids 17-178 of mature p75 TNF receptor, not amino acids 1-162 as stated in the Appeal Brief. Appellants thank the Examiner for this observation.

The diagram also depicts observations with respect to minimum amino acid sequence needed for TNF-binding activity that are discussed above in section III.A.3. Appellants provided evidence that a fragment consisting of amino acids 1-162 of p75 TNF receptor bound TNF, but truncating the C-terminus to amino acid 142 destroyed TNF-binding activity. Thus, as depicted in the top cross-hatched box of the diagram, some portion of amino acids 142-162 is required for binding TNF. The diagram further depicts the Examiner's assertion that Chan (2000) [Appendix B-76] teaches that some portion of amino acids 10-54 at the N-terminus is required for binding TNF. See the second cross-hatched box of the diagram.



5. Written description support for scope of the claims

When the claims are properly construed, the genus of TNF-binding soluble fragments of p75 TNF receptor is not the "vast genus" envisioned by the Examiner. The Answer agrees that "soluble fragments" include the extracellular domain as well as fragments thereof that specifically bind TNF. See page 34 (1st full paragraph) of Answer. Since mutated variants need not be considered, for the reasons discussed above, the number of possible TNF-binding fragments of the extracellular domain is relatively small. A TNF-binding soluble fragment must contain at least amino acids 54-142 of p75 TNF receptor. Some portion of amino acids 10-162 is required for specifically binding TNF, as recited in the claims.

The representative species distributed throughout the breadth of the claim, as depicted in the corrected diagram above, provide sufficient written descriptive support for the scope of the claims. Moreover, the knowledge in the prior art regarding the location of the TNF-binding region at amino acids 17-178 provides sufficient structure-function correlation to support written description of the scope of the claims. Taken together, the available information on representative species and structure-function relationship more than amply support the limited breadth of the claims.

There are representative species throughout the scope of the claim that can be found in the specification and the prior art. For example, the specification discloses immunoglobulin fusion proteins comprising the entire extracellular domain of TNF receptor. Factual evidence in the Lyman Declaration, paragraph 17 [Appendix B-143], confirms that the skilled artisan would have understood at the time that "[a]lthough the working examples exemplify a fusion protein comprising the entire extracellular region of the 55 kd TNFR, it is readily apparent that the application's description applies equally to the 75 kd TNFR."

Moreover, the Answer admits that both Dembic and Smith (1990) disclose a fragment consisting of the entire extracellular domain of p75 TNF receptor. Pages 11-12 of Answer. This extracellular domain extends from amino acids 1 to 235, as illustrated in the first representative species depicted in the diagram above. The Answer states at page 46 that "Dembic and Smith generally teach that the TNF-binding region of the insoluble receptor is found in particular regions of the extracellular domain . . ." The Examiner agrees that as of

the effective filing date, the skilled artisan would have expected this extracellular domain to bind TNF. Page 34 (1st full paragraph) of Answer.

The second line depicted in the diagram above is the putative TNF binding site referenced in Smith (1990) at amino acids 17-178. Sentence spanning pages 1020-1021 and page 1021, 3rd col. of Smith (1990) [Appendix B-211]. The identification of this binding site thus provides further species of fragments that are truncated at the N-terminus at positions up to residue 17, as well as species of fragments that are truncated at the C-terminus at positions down to residue 178. Appellants provided evidence that fragments truncated at the C-terminus at positions down to residue 162 retain TNF binding activity and are thus TNF-binding soluble fragments.

Dembic [Appendix B-80] provides another species that is a soluble fragment with TNF binding activity, commencing with amino acid 5. Appellants note that while the question marks "??" indicate that the exact C-terminal amino acid is unknown, the important information is that yet another species of TNF-binding soluble fragment, truncated at the N-terminus at positions up to residue 5, was known in the art.

Thus, the specification and prior art disclose representative species distributed throughout the scope of the claim. Federal Circuit precedent permits Applicants to rely on this knowledge in the prior art, analogous to the situation in *Falkner v. Inglis*. The written description requirement is fully met by the disclosed representative species.

There is also adequate written description because there is a known correlation between structure and function. Smith (1990) at pages 1020-1021 identifies the likely TNF binding site as being within amino acids 17-178. Thus, the common structural features of the claimed genus were known in the art at the time of filing, as was the correlation between these structural features and function. Such a correlation known in the art satisfies the written description requirement. *See Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332, 65 U.S.P.Q.2d 1385, 1398 (Fed. Cir. 2003) (the written description requirement may be satisfied "if *in the knowledge of the art* the disclosed function is sufficiently correlated to a particular, known structure") (emphasis added).

6. The written description rejection of each of claims 103, 107, 111 and 128

Appellants' written descriptive support is even more compelling for claims where the soluble fragment includes the N-terminal amino acid. Claims 103, 107, 111 and 128 require that the "soluble fragment" comprise SEQ ID NO: 10, which corresponds to amino acids 1-18 of the p75 TNF receptor. For these claims, the scope of the term "soluble fragment" is an even more limited group of fragments ranging from at least amino acids 1-142 to at most amino acids 1-235. Thus, the representative species and known structure-function relationship described above is more than ample support for the reduced breadth of these claims. Written description for each of these claims must be considered separately from claims 62, 102, 105, 110, 113, 114, 119, 120, 123, 124, 129, 130, 132, 133 and 137 because the patentability considerations differ from those of the latter claims.

7. The written description rejection of claim 135

Claim 135 also requires that the soluble fragment include the N-terminal amino acid. Thus, patentability of this claim must be considered separately. The claim recites that the "soluble fragment" comprises SEQ ID NO: 10, which corresponds to amino acids 1-18 of the p75 TNF receptor. In claim 135, the scope of the term "soluble fragment" is an even more limited group of fragments ranging from at least amino acids 1-142 to at most amino acids 1-235. The representative species and known structure-function relationship described above is more than ample support for the breadth of claim 135.

Moreover, as noted in the Appeal Brief, claim 135 recites the language "consisting of," and thus is narrower in scope than a claim reciting "comprising." The reduced breadth further weakens the Examiner's assertion of undue breadth.

Finally, the fusion protein of claim 135 is specifically illustrated by the working example of a fusion protein consisting of a soluble fragment of a TNF receptor fused to a fragment of a human IgG heavy chain constant region lacking the CH1 domain. See Example 11, pages 42-43 of the specification. While this fusion protein was exemplified for p55 TNF receptor, the disclosure applies equally to the p75 TNF receptor for the reasons discussed above in paragraph 11 of the Lyman Declaration [Appendix B-143] and the Federal Circuit's *Falkner* case.

For all of the reasons described above and in the Appeal Brief, there is adequate written specification of TNF-binding soluble fragments of p75 TNF receptor

B. The obviousness rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 125-131 and 134-137 under 35 U.S.C. § 103 over Dembic *et al,* Cytokine 2: 231-237, 1990 in view of Capon (US Patent No. 5,116,964)

1. Teaching away

There is no dispute that "the immunoglobulin heavy chain constant region has pro-inflammatory activity that is retained by fusion proteins of such, or that soluble fragments of TNFR could act as an anti-inflammatory agent." Page 57 (2nd paragraph) of Answer. Thus, the ordinary skilled artisan would have been discouraged from combining an anti-inflammatory agent, such as soluble fragments of p75 TNF receptor disclosed in Dembic, with the pro-inflammatory constant region of an immunoglobulin heavy chain disclosed in Capon. The Examiner presented no direct response to this argument and apparently accorded it little or no weight.

In support of his obviousness rejection, the Examiner relied on an asserted *in vitro* use to affinity purify TNF as allegedly motivating the combination. That weak rationale is inadequate to support a *prima facie* case, for the reasons discussed below in III.B.2.

"[W]hen the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 416, 127 S. Ct. 1727, 1740, 82 U.S.P.Q.2d 1385, 1395 (2007), citing *United States v. Adams*, 383 U.S. 39, 51-52, 86 S. Ct. 708, 714-715, 148 U.S.P.Q. 479, 483 (1966). See also *In re Beattie*, 974 F.2d 1309, 1313, 24 U.S.P.Q.2d 1040, 1042-43 (Fed. Cir. 1992) (evidence that art teaches away from the invention should be considered).

In *U.S. v. Adams*, Adams' battery was deemed nonobvious and patentable despite the fact that each of the elements of the Adams battery was well known in the art. The Supreme Court pointed to "factors [that] when taken together, would, we believe, deter any investigation into such a combination as is used by Adams." *Adams,* 86 S. Ct. at 714.

Similarly, in this case, the fact that the TNF receptor soluble fragment is an anti-inflammatory component and the immunoglobulin heavy chain constant region is a pro-inflammatory component would deter investigation into such a combination. Moreover, as discussed below in section III.B.3., the resulting combination exhibits unexpected results that were completely different from what was expected based on the predicted functions of these components. Such unexpected results *per se* necessitate a finding of nonobviousness.

2. *The asserted in vitro use does not motivate construction or selection of the claimed fusion protein*

The Examiner's proposed motivation for combining a TNF receptor fragment and an immunoglobulin fragment disclosed in Capon is *in vitro* use for affinity purifying TNF. The Examiner has not pointed out any way in which such a fusion protein is more functional than a TNF receptor soluble fragment alone as an affinity purification reagent for TNF. Moreover, Capon discloses hundreds or even thousands of variations of immunoglobulin fragments. The Examiner's alleged motivation fails to supply an adequate rationale for selecting fusion to "all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region" as recited in the claims. See pages 56-59 of Answer.

The proposed use of TNF receptor fusion proteins for purifying TNF through affinity purification is based on the ability of TNF receptors to bind TNF. The TNF receptor fusion protein would presumably be chemically attached to a matrix and exposed to a solution containing TNF. Binding of the TNF molecule to the TNF receptor would then permit separation of the TNF from contaminants. Thus, the primary functional element for purposes of affinity purification is the TNF receptor fragment, and the extent or identity of fused sequence from the immunoglobulin would be largely irrelevant for such a purpose.

The Examiner has provided no reason why one would be motivated in particular to fuse "all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region" to a soluble TNF binding protein for the purpose of affinity purification. Suitable fusions for this use might contain (1) no added sequence at all, (2) any portion of a non-immunoglobulin peptide, (3) any portion of an immunoglobulin, or (4) any portion of the constant region of an immunoglobulin, *e.g.*,

CH1 domain alone, hinge domain alone, CH2 domain alone, CH3 domain alone, a fragment of any of these domains, or some combination thereof.

The selection of a species from a large genus disclosed in a prior art reference is nonobvious. *See In re Baird*, 16 F.3d 380, 382-83, 29 U.S.P.Q.2d 1550, 1552 (Fed. Cir. 1994).

Capon contemplates hundreds or even thousands of variations of immunoglobulin fragment fusion proteins, including variations containing CH1. *See, e.g.,* cols. 12-14 of Capon U.S. Patent No. 5,116,964 [Appendix B-26]. Accordingly, the *in vitro* affinity purification rationale proposed by the Examiner applies to a huge genus of possible fusion proteins containing immunoglobulin fragments of varying lengths and with varying conformations, including monomeric, homodimeric, heterodimeric, trimeric, tetrameric, homomultimeric and heteromultimeric forms. The Examiner has not provided any reasoning supporting why, for *affinity purification,* one of ordinary skill in the art would have selected the particular immunoglobulin fragments and conformation as presently claimed. While the Examiner points to one selected embodiment among many, no disclosure or reasoning ties that particular embodiment to affinity purification.

However, the ordinary skilled person would have been *discouraged* from selecting a fusion protein containing all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain. A fusion protein containing the hinge, CH2 and CH3 domains inherently has a homodimeric structure due to disulfide bonding at the hinge region. Knowing that TNF is found in a trimeric form, the ordinary skilled person might have been more motivated to choose a ***trimeric*** TNF receptor fusion structure (*see, e.g.*, trimeric structures at col. 13 of Capon) to bind to ***trimeric*** TNF, rather than a homodimeric structure.

Alternatively, knowing that the structure of the binding site on the TNF receptor was unknown (see page 17 of Final Action), the ordinary skilled person might have preferred a ***monomeric*** form of TNF receptor fusion rather than a dimeric form. The monomeric form might have been the first choice for successful binding, because a dimeric form might not bind as well or at all. Appellants presented unrebutted evidence that a dimeric form could have had a spatial geometry that was *unable* to bind to the trimeric TNF ligand. See Lesslauer Declaration A [Appendix B-129].

Such factors which discourage the claimed combination must be considered. See, e.g., *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 417, 127 S. Ct. 1727, 1740, 82 U.S.P.Q.2d 1385, 1395 (2007); *United States v. Adams*, 383 U.S. 39, 51-52, 86 S. Ct. 708, 714-715, 148 U.S.P.Q. 479, 483 (1966). The Examiner has thus failed to articulate the required rationale explaining specifically why the cited art motivates the claimed invention, which comprises "all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region." The Supreme Court has held that "rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *KSR Int'l. Co. v. Teleflex, Inc.*, 550 US. 398, 418, 127 S. Ct. 1727, 1741, 82 U.S.P.Q.2d 1385, 1396 (2007).

3. No reasonable expectation of success

In the Final Action, the Examiner had asserted a reasonable expectation of success that the claimed *dimeric* TNF receptor fusion proteins would bind *trimeric* TNF, based on a citation of Smith and Baglioni (1989) as allegedly showing that TNF receptors contain at least two subunits. The Answer concedes in the first paragraph of page 60 that Appellants' argument "successfully weakens Smith and Baglioni as evidence in support of a reasonable expectation of success" since Smith and Baglioni do not clarify which receptor subunits are found in their observed multimeric complexes. Appellants noted that, even a decade later, Chan (2000) [Appendix B-76] suggests that *trimers* of TNF receptors are a favored conformation.

One of ordinary skill in the art, knowing that TNF is a trimeric ligand, would not have had a reasonable expectation that a dimeric form of p75 TNF receptor soluble fragment fusion proteins would bind TNF. Appellants provided unrebutted evidence, Lesslauer Declaration A [Appendix B-129], which states that "the spatial geometry of the receptor binding site was unknown. Thus, it could have been possible that the fusion with IgG fragments created a spatial structure that . . . was *completely unable to bind TNFα [emphasis added.]*"

The Examiner erroneously maintains that "in view of *KSR* an obviousness rejection under U.S.C. § 103 does not actually require a test of a 'reasonable expectation of success.'" Page 60, 2nd paragraph of the Answer. However, expectation of success is clearly

an important factor given KSR's emphasis on "predictable solutions" and "anticipated success" and "predictable use" of prior art elements. *KSR, supra,* 550 U.S. at 421, 127 S. Ct. at 1742 82 U.S.P.Q.2d 1385, 1397; 550 U.S. at 417, 127 S. Ct. at 1740 82 U.S.P.Q. at 1396. KSR also cites *United States v. Adams, supra,* with approval. In *United States v. Adams*, the prior art's lack of a reasonable expectation of success led to the Supreme Court's holding of nonobviousness.

The Examiner's position regarding a reasonable expectation of success is manifestly noncompliant with the U.S. Patent and Trademark Office's (USPTO) own protocols and guidelines. Indeed, section 2143.02 of the Manual of Patent Examination and Procedure is entitled "**Reasonable Expectation of Success Is Required**" and cites to *KSR*. *Each* of the rationales advanced by the USPTO for obviousness rejections requires a specific finding of predictable results and/or a reasonable expectation of success. Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.*, 72 Fed. Reg. 57526-35. Failure to follow the agency's own rules is arbitrary, capricious and an abuse of discretion. See 5 U.S.C § 706, the Administrative Procedure Act.

The Answer now attempts to rely on the fact that a soluble TNF receptor fragment described in Dembic binds TNF as support for an expectation that Appellants' claimed fusion proteins would bind TNF as well. See paragraph spanning pages 60-61 of the Answer. However, Appellants' evidence proves this is pure speculation. Lesslauer Declaration A [Appendix B-129] states that it was possible that the spatial geometry of the multimeric receptor would not accommodate the trimeric TNFα and thus would be unable to bind to it. Therefore, the TNF-binding capabilities of the soluble fragment portion alone were an inadequate basis for predicting that the dimeric fusion protein would have bound TNF.

In view of Appellants' unrebutted evidence of unpredictability, the discouragement in the cited art for using a dimeric form, and the conceded weakness of the evidence supporting the possibility of success, the Examiner's assertion of a reasonable expectation of success is not supported by the record.

4. New properties and functions that are completely different from the predicted properties and functions require a finding of nonobviousness for the claimed combination

The Answer admits that Appellants' evidence shows unexpected results, in six different categories, for two different fusion proteins falling within the scope of the present claims. Results were shown for a fusion of a soluble fragment of human p75 TNF receptor with all of the domains of the constant region of a human IgG1 heavy chain other than the first domain, and for a fusion of a soluble fragment of human p75 TNF receptor with all of the domains of the constant region of a human IgG3 heavy chain other than the first domain. Nevertheless, the Examiner accords these unexpected results no weight in making his obviousness determination because "there is no conception in the specification" of these "particular species of fusion protein containing the full-length extracellular domain of the 75 kD human TNF receptor". Nearly identical quotations are found at page 63 (1nd full paragraph), at page 63 (3rd paragraph), at page 64 (1st paragraph), and at page 65 (1st paragraph) of the Answer.

The unexpected nature of Appellants' results is not disputed. The acknowledged unexpected results show that the combination of the two elements in the fusion protein, *i.e.* the TNF receptor portion, and the immunoglobulin portion, is not carrying out the function that would be predicted for each element separately. As explained in the Appeal Brief at pages 48-55, it was the expectation in the art that the immunoglobulin portion of the claimed fusion protein would retain the pro-inflammatory effector function of immunoglobulins. Contrary to this predicted function, Appellants provided evidence showing that the pro-inflammatory effector function of the fusions was drastically reduced. The Examiner agreed that it was unexpected to see the drastically reduced effector function in the form of reduced or absent complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), as well as the failure to form the aggregating complexes that precede activation of effector function. See page 63 of Answer. The Examiner also agreed that the increase in TNF neutralizing potency (page 64), the increased kinetic stability (page 65) and the improved inhibition of TNF (page 65) were each unexpected results.

The undisputedly unexpected results show that the combination of elements is not carrying out the function predicted for the elements separately. This fact mandates a

finding of nonobviousness in view of the Supreme Court's statement that: "A court must ask whether the improvement is more than the predictable use of prior-art elements according to their established functions." *KSR Inter. Co. v. Teleflex Inc.* 550 U.S. 398, 417, 82, 127 S. Ct. 1727, 1740, 82 U.S.P.Q.2d 1385, 1396 (2007). In this case, the improvement is clearly more than the predictable use of a prior art element according to its established function.

The Examiner committed reversible legal error by refusing to weigh the admittedly unexpected properties of the claimed product in making his obviousness rejection. The Examiner must consider the unexpected biological activity of a product even when the chemical structure or composition of the product is allegedly structurally obvious. *In re Sullivan,* 498 F.3d 1345, 1352, 84 U.S.P.Q.2d 1034, 1039 (Fed. Cir. 2007). In *Sullivan*, the Board affirmed an obviousness rejection of claims directed to an antibody fragment composition without considering three expert declarations concerning unexpected results. The Board refused to consider the evidence on the ground that it was only relevant to the intended use of the claimed composition. The Federal Circuit held that the Board erred by failing to consider the declarations in a meaningful way.

New, completely different biological properties can render a claimed combination nonobvious. See *Application of Papesch*, 315 F.2d 381, 137 U.S.P.Q. 43 (C.C.P.A. 1963). In *Papesch*, the appellant argued that the showing of unpredictable and "completely dissimilar biological properties" of a representative compound established the patentability of the genus of compounds he claimed. *Id.* at 383. The appellate court reversed the Board's finding of obviousness in its entirety based on these unexpected results, because the Board's decision "rest[ed] on one fundamental error of law, namely, the failure to take into consideration the biological or pharmaceutical property of the compounds . . . on the ground that to chemists the structure of the compounds would be so obvious as to be beyond doubt." *Id.* at 391. *Application of May*, 574 F.2d 1082, 197 U.S.P.Q. 601 (C.C.P.A. 1978) (unexpected lack of addictive properties satisfactorily rebutted the presumed expectation that structurally similar compounds have similar properties); *Application of Henderson*, 348 F.2d 550, 554, 146 U.S.P.Q. 372 (C.C.P.A. 1965) (composition containing two known components was patentable in view of its unexpected property even though the combination of the components was motivated).

In the present case, as in *Papesch*, the Examiner has committed a fundamental, reversible legal error by ignoring the unexpected properties of the claimed compounds. The error is particularly problematic when the alleged *prima facie* case of obviousness is weak. The Examiner conceded that the reasonable expectation of success was "weaken[ed]" as discussed above in III.B.3. Even the allegation of structural obviousness is based on a weak assertion that such a structure might be a possibility among many hundreds of potential combinations, for reasons discussed above in III.B.2. Thus, controlling precedent requires that the Examiner consider unexpected properties of the claimed compounds. When considered, these numerous, admittedly unexpected properties render the claimed fusion proteins nonobvious.

The fact that Appellants' claimed fusion proteins behave in a completely unexpected manner compared to the predicted function of the component elements proves that Appellants' claimed compounds are clearly "more than the predictable use of prior art elements according to their established functions." *KSR, supra*, 127 S. Ct. at 1731, 82 U.S.P.Q.2d at 1396. Moreover, the fact that "the elements worked together in an unexpected and fruitful manner support[s] the conclusion that [the claimed] design was not obvious to those skilled in the art. *KSR, supra*, 127 S. Ct. at 1740, 82 U.S.P.Q. at 1395 (discussing *U.S. v. Adams, supra*). The unexpected behavior of these elements in combination outweighs any *prima facie* case of obviousness, particularly when the *prima facie* case is conceded to be weak.

5. The obviousness rejection of claims 114 and 137

The asserted motivation to make the claimed fusion protein to affinity purify TNF completely fails to apply to dependent claims 114 and 137. These claims recite a "pharmaceutical composition" comprising a "pharmaceutically acceptable carrier material." Non-sterile and possibly toxic or contaminated solutions are not pharmaceutically acceptable carrier materials. Similarly, affinity purification compositions are not "pharmaceutical compositions."

Affinity purification is described in Capon as involving derivatization and cross-linking to a matrix. See Capon at col. 22, lines 1-6 [Appendix B-26]:

> Derivatization with bifunctional agents is useful for preparing intermolecular aggregates of the hybrid immunoglobulin with polypeptides as well as for cross-linking the hybrid immunoglobulin to a water insoluble support matrix or surface for use in the assay or affinity purification of its ligands.

In the Final Action, the Examiner had stated that sterile and isotonic solutions meet the definition of a "pharmaceutically acceptable carrier" as recited in claims 114 and 137:

> Capon further teaches placement of the purified hybrid immunoglobulin in *"sterile, isotonic formulations"* that are "preferably liquid" and "ordinarily a physiologic salt solution" (col. 31, lines 4-8). Such ***solutions meet the definition*** *of a "pharmaceutically acceptable carrier material"* (as in claim 114). [Page 15 of Final Action, emphasis added.]

In a misguided attempt to link use as an affinity purification with Capon's disclosure of sterile and isotonic formulations described above, the Examiner argued that the ordinary skilled person would be motivated to include the fusion protein in a pharmaceutically acceptable carrier material "[a]s described above" in the preceding quotation "in order to resuspend the hybrid immunoglobulin for use following purification." Paragraph bridging pages 15-16 of Final Action. The Examiner failed to provide logical scientific reasoning or any factual basis for stating that proteins destined for use in affinity purification, including chemical cross-linking to a matrix as described by Capon, should be resuspended in sterile and isotonic formulations. Appellants questioned the Examiner's factual assumption and challenged the Examiner to provide supporting evidence.

The Examiner had no response to Appellants' challenge. Instead, the Examiner now asserts that the claim broadly encompasses both sterile and non-sterile compositions. As an example, the Examiner asserts that water can be "orally administered" without being sterile or isotonic, and that proteins placed in water meet the language of claims 114 and 137. Page 68 of Answer.

It is well known that pharmaceutical compositions comprising a protein are administered parenterally by injection and must be sterile. See, *e.g.*, Ruddle, *et al., J. Exp. Med.,* 172: 1193-1200 (1990) [Appendix B-494], which describes injection of proteins, and other generally contemporaneous publications, such as Ashkenazi *et al., Proc. Natl Acad.*

Sci., 88:10535-10539 (1991) [Appendix B-507], Mohler *et al.*, *J. Immunol.*, 151:1548-1561 (1993) [Appendix B-181]; Mori *et al.*, *J. Immunol.,* 147: 3178-82 (1996) [Appendix B-512], and Piguet *et al.*, *J. Immunol.,* 77(4):510-514 (1992) [Appendix B-512].

This well established knowledge is confirmed by statements in art of record. For example, prior art U.S. Patent No. 4,894,439 describes production of compositions containing proteins for parenteral use. It teaches that they are "formulated with sterile ingredients compounded and packaged aseptically." Col. 20, lines 60-62 of U.S. Patent No. 4,894,439 [Appendix B-517]. This patent also describes in Example XVIII that the "[f]inal purification [protein] product is suspended in Sterile Water for Injection, U.S.P." Col. 20, lines 10-11. A contemporaneous patent issued October 23, 1990 confirms that compositions for parenteral administration must be sterile: "Effective amounts of the [compounds in question] can be administered to a subject by any one of various methods, for example, orally as in capsule or tablets, or parenterally in the form of sterile solutions." U.S. Patent No. 4,965,271, col. 10, lines 16-19 [Appendix B-534]. This patent goes on to state that solutions or suspensions for parenteral administration can include sterile diluents and other additives such as antibacterial agents or agents for the adjustment of tonicity. Col. 11, lines 14-23

The Examiner's assertion that an injectable pharmaceutical composition comprising an immunosuppressive protein need not be sterile is wrong. The assertion is senseless from a real world point of view, as one of skill in the art would readily appreciate. Beyond that, sterility of a biologic, such as a protein, is mandated by law. (*see, e.g.,* 21 C.F.R. §610.12). Any factual finding to the contrary would be clearly erroneous. Thus, the claimed "pharmaceutical" compositions do not and cannot encompass non-sterile water as alleged by the Examiner. Page 68 of Answer.

The Examiner impermissibly ignored the recitation of "pharmaceutical composition" in the preamble, alleging that it was merely an intended use. Page 68 of Answer. The Examiner's claim construction is legally erroneous for two independent reasons. First, a claim's preamble is a meaningful limitation when it results in a physical difference in the product claimed. *See In re Stencel*, 828 F.2d 751, 754, 4 U.S.P.Q. 2d 1071, 1073 (Fed. Cir. 1987).

In *Stencel,* the Federal Circuit held that the function recited in the preamble limited the claimed drivers to those with a structure "suitable for use" according to the recited

function. In *Stencel*, Appellants were claiming a driver comprising certain elements. The driver's function and mechanism of operation was described in the preamble, but not in the body of the claim. Although the Board agreed with Stencel that there was a fundamental difference between the mechanism recited in the preamble and that disclosed in the prior art, the Board rejected the claims because it ignored the functional description of the driver.

The Federal Circuit reversed, stating that the mechanism of operation described in the preamble did in fact limit the structure of the collar and distinguished the prior art:

> . . . appellant is not barred from describing the driver in terms of the structure imposed upon it by the collar having plastically deformable lobes. *The framework – the teachings of the prior art – against which patentability is measured is not all driver broadly, but drivers suitable for use in combination with this collar, for the claims themselves are so limited. Stencel*, 828 F.2d at 754 (emphasis added).

Similarly, in the present case, the preamble limits the physical characteristics of the composition such that it is suitable for pharmaceutical use. In particular, the composition must be sterile.

Second, a claim's preamble is a meaningful limitation when it distinguishes the prior art. *See Catalina Marketing,* 289 F.3d at 808-809, 62 U.S.P.Q. at 1785-86, citing *Bristol-Myers Squibb Co. v. Ben Venue Labs., Inc.*, 246 F.3d 1368, 1375, 58 U.S.P.Q.2d 1508, 1513 (Fed. Cir. 2001) (A preamble may limit when employed to distinguish a new use of a prior art apparatus or process.); *Kropa v. Robie*, 187 F.2d 150, 88 U.S.P.Q. 478 (C.C.P.A. 1951).

In *Kropa,* the court read the preamble as a meaningful limitation because not all of the prior compositions were capable of the use in the preamble. Although the context was an interference, priority depended on whether the preamble should be given patentable weight as a limitation. A representative claim recited: "An abrasive article comprising abrasive grains and a hardened binder . . ." *Id.* at 151. Appellants argued that the claims should be read to cover any combination of abrasive grains with the binders, despite the fact that some combinations did not form abrasive articles. The court disagreed, holding that the phrase "abrasive article" in the preamble was in fact a claim limitation because "[e]very union of substances capable inter alia of use as abrasive grains and a binder is not an

'abrasive article.'" *Kropa,* 187 F.2d at 152. Similarly, in the present case, not every composition containing a TNF receptor-immunoglobulin fusion protein and a carrier is capable of use as a pharmaceutical composition, particularly compositions that are not sterile. Thus, the preamble of claims 114 and 137 must be given patentable weight because it requires a physical difference in the composition, *e.g.*, sterility.

Appellants' specification states that a pharmaceutical composition is for therapeutic purposes (see, *e.g.,* page 12, lines 10-20), and states that such pharmaceutical preparations should contain "non-toxic, inert, therapeutically compatible carrier materials." Page 12, lines 10-15 of Specification. Elsewhere, the specification states that these "pharmaceutical preparations" are for treatment of illnesses involving TNF:

> The TNF-BP in accordance with the invention as well as their physiologically compatible salts, which can be manufactured according to methods which are known in the state of the art, can also be used for the production of *pharmaceutical preparations, primarily those for the treatment of illnesses in which TNF is involved in their course*. Page 20, lines 12-18 [emphasis added].

In contrast, the term "pharmaceutical preparation" is never used in the immediately preceding paragraphs discussing other *in vitro* uses for the TNF binding proteins, such as use as an antigen to generate monoclonal antibodies or use in detection assays. Page 19, line 20, to page 20, line 2 of the Specification.

The specification's disclosure is consonant with the use of the term in the art. For example, *Takeda Pharmaceutical Co. Ltd. v. Teva Pharmaceuticals USA Inc.*, 542 F. Supp. 2d 342, 348 (D. Del. 2008) indicates that plain and ordinary meaning of "pharmaceutical composition," in the absence of an explicit definition in the specification, means "a medicinal drug product *in a state suitable for administration to a patient* (emphasis added)."

Capon teaches that its polypeptides when prepared for parenteral administration, *e.g.* through intravenous delivery, should be sterile and isotonic:

> The novel polypeptide is placed into *sterile, isotonic* formulations together with required cofactors, and optionally are administered by standard means well known in the field. The formulation is preferably liquid, and is ordinarily a

> physiologic salt solution containing 0.5-10 mM calcium, non-phosphate buffer at pH 6.8-7.6, or may be lyophilized powder.
>
> It is envisioned that *intravenous delivery*, or delivery through catheter or other surgical tubing will be the primary route for therapeutic administration.
>
> Capon, col. 31, lines 4-13 [Appendix B-26].

This understanding in the art is underscored by the Examiner's prior positions in the Non-Final Office Action dated April 3, 2006 (see page 11) and the Final Action (page 15) that a "sterile" and "isotonic" formulation is a "pharmaceutically acceptable carrier material. *See also, Synthetic Patents Co., Inc. v. United States*, 11 Cust.Ct. 157, 160-162, 1943 WL 4396 (Cust.Ct. 1943)[5] (parenteral administration, *i.e.*, "giv[ing] something underneath the skin," requires sterility as mandated by law).

Thus, Appellants' specification teaches that the pharmaceutical compositions described are for administration of the protein to patients, *e.g.* to treat illnesses involving TNF. Even the art cited by the Examiner, Capon, teaches that sterile and isotonic compositions are to be used for intravenous delivery of protein. The asserted affinity purification compositions are clearly *not suitable* for administration to patients.

Affinity purification compositions would be prepared under non-sterile laboratory conditions in chemical reactions to link the protein to a matrix, and which would contain potentially toxic or painful additives. Capon describes such compositions as prepared via cross-linking by chemical agents, including derivatization with bifunctional agents. It is scientifically unreasonable for the Examiner to assert, without evidence, that a non-sterile and possibly toxic composition used for affinity purification could be a pharmaceutical composition, or that the chemical solution involved would be a therapeutically compatible carrier.

The present case is even more compelling than *Kropa, supra.* The holding in *Kropa* was based on the fact that not every prior composition containing abrasive grains and

[5] In this case the court based its decision on witness testimony that included the following: "they are merely those necessary to make any medicinal suitable for parenteral administration; in other words, that they are those processes by which it is made possible to give something underneath the skin. So there are two things that are done: the establishment of sterility, of activity for purposes, and those are required by law, and secondly, the making it possible to for the physician to administer it under the skin."

a binder produced an abrasive article. In this case, no typical affinity purification composition would be capable of use as a pharmaceutical composition. The present case is also highly analogous to *Stencel, supra*. In *Stencel*, the Federal Circuit held that the function recited in the preamble was a meaningful limitation because it limited the structure of the claimed driver. Similarly, here, the pharmaceutical use recited in the preamble is a meaningful limitation because it limits the physical characteristics of the composition claimed.

Even if the preamble is not taken as limiting, claims 114 and 137 require inclusion of a pharmaceutically acceptable carrier. The use of the term pharmaceutically acceptable vehicle as meaning suitable for administration to patients is consistent with the use of similar terms in other patent applications. For example, the Capon reference cited by the Examiner states that pharmacologically acceptable vehicles are for administration to patients:

> The novel compositions provided herein are purified and *formulated in pharmacologically acceptable vehicles for administration to patients*. . . Col. 6, lines 6-10 [Appendix B-26].

For these reasons alone, the rejection of pharmaceutical composition claims 114 and 137 as obvious in view of solutions of p75 TNF receptor fusion proteins for affinity purification must be reversed. In addition, for the reasons discussed previously with respect to the independent claims, the claims directed to the underlying fusion proteins themselves are nonobvious.

C. The new matter rejection of claims 140-144

At page 75, the Answer states "the new matter rejection was not based on the grounds that N227 contains more sequence tha[n] displayed in Figure 4, but rather because claim 140 requires a 'cDNA insert' and there is no cDNA described in the specification that encodes the full-length 75 kD TNFR[]." The rejection appears to be based on the pervasive misconception that the specification does not describe cDNA encoding full length p75 TNF receptor simply because Figure 4 does not display the full sequence, despite the undisputed facts that Appellants (1) actually reduced to practice a cDNA encoding the full length p75 TNF receptor (as recorded in the Dembic publication), (2) described uniquely identifying physical characteristics, including N-terminal and internal peptide sequences, and (3) added to the specification a citation to a publication disclosing the complete sequence, Smith

(1990). For all of the reasons described above in section III.A. with respect to written description, this rejection is legally and factually erroneous.

The Answer also argues that reference to the deposit is improperly inserted in the phrase "DNA sequences that code for insoluble [] fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD." Page 74 of the Answer. The Examiner alleges that the deposit reference is inappropriate here because the described encoded "insoluble fractions" constitute a genus that is "not limited to any particular sequence and thus encompasses any variant with one or more amino acid changes that retains TNF-binding and the recited weight." Page 74 of the Answer.

This argument is unpersuasive in view of the factually parallel case *Evans Med. v. American Cyanamid Co.,* 11 F.Supp.2d 338 (S.D.N.Y. 1998), and the Federal Circuit decision that affirmed, *Evans Med. v. American Cyanamid Co.,* 215 F.3d 1347 (Fed. Cir. 1999) (non-precedential). In *Evans*, the Federal Circuit approved applicants' insertion of deposit information for a particular hybridoma cell line on the strength of a disclosure in the original specification which read: "a monoclonal immunoglobulin specific for ACAP." *Evans,* 11 F.Supp.2d at 362. This phrase describes a vast genus of possible monoclonal antibodies specific for ACAP, yet applicants were permitted to deposit a particular hybridoma that secretes a single monoclonal antibody out of this vast genus. Similarly, in the instant case, insertion of deposit information for DNA encoding a particular insoluble TNF binding protein is consistent with *Evans.*

Thus, for all the reasons discussed above and in the Appeal Brief, the rejection of claims 140-144 for new matter is factually and legally erroneous.

IV. CONCLUSION

The Examiner's legal and factual errors thus necessitate reversal of all rejections and return of this case to the Examiner for appropriate allowance of the claims.

Dated: May 26, 2009

Respectfully submitted,

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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 08/444,790

62. A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10); and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

102. The protein of claim 62, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

103. The protein of claim 102, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

105. The protein of claim 62, wherein said human immunoglobulin IgG heavy chain is IgG_1.

106. A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-

polyacrylamide gel, and (iii) comprises the amino acid sequences LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), LCAP (SEQ ID NO: 12), VFCT (SEQ ID NO: 8), NQPQAPGVEASGAGEA (SEQ ID NO: 9) and VPHLPAD (SEQ ID NO: 13),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8); and

(b) all of the domains of the constant region of a human IgG_1 heavy chain other than the first domain of the constant region;

wherein said protein specifically binds human TNF.

107. A recombinant protein encoded by a polynucleotide which comprises two nucleic acid subsequences,

(a) one of said subsequences encoding a human TNF-binding soluble fragment of an insoluble human TNF receptor protein having an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, said soluble fragment comprising the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) the other of said subsequences encoding all of the domains of the constant region of the heavy chain of a human IgG immunoglobulin other than the first domain of said constant region,

wherein said recombinant protein specifically binds human TNF.

110. The protein of claim 107, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

111. The protein of claim 110, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

113. The protein of any one of claims 107, 110 or 111, wherein said human immunoglobulin heavy chain is IgG_1.

114. A pharmaceutical composition comprising the recombinant protein of any of claims 62, 107, 134 or 135 and a pharmaceutically acceptable carrier material.

119. The protein of claim 62, wherein the protein is purified.

120. The protein of claim 62, wherein the protein is produced by CHO cells.

121. The protein of claim 62, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of the constant region.

123. The protein of claim 62, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

124. The protein of claim 105, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hγ1 vector (deposited at Deutschen Sammlung von

Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

125. The protein of claim 106, wherein the protein is purified.

126. The protein of claim 106, wherein the protein is produced by CHO cells.

127. The protein of claim 106, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG_1 heavy chain other than the first domain of the constant region.

128. The protein of claim 106, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

129. The recombinant protein of claim 107, wherein the recombinant protein is purified.

130. The recombinant protein of claim 107, wherein the recombinant protein is produced by CHO cells.

131. The recombinant protein of claim 107, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG_1 heavy chain other than the first domain of the constant region.

132. The protein of claim 107, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

133. The protein of claim 113, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by the DNA insert of pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

134. A protein consisting of

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, and (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO:12) and VFCT (SEQ ID NO:8), and

(b) all of the domains of the constant region of a human IgG_1 heavy chain other than the first domain of the constant region,

wherein the protein specifically binds human TNF, and

wherein the protein is produced by CHO cells.

135. The protein of claim 134, wherein the soluble fragment comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

136. The protein of claim 134, wherein the protein is purified.

137. A pharmaceutical composition comprising the recombinant protein of claim 105 and a pharmaceutically acceptable carrier material.

140. A protein comprising

(a) human tumor necrosis factor (TNF) binding soluble fragment of the amino acid sequence encoded by the cDNA insert of the plasmid deposited with the ATCC on October 17, 2006 under accession number PTA 7942,

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

141. The protein of claim 140 consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

142. The protein of claim 140

wherein the protein is expressed by a mammalian host cell.

143. The protein of claim 142, wherein the mammalian host cell is a CHO cell.

144. The protein of claim 142 consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

APPENDIX B

Evidence
Table of Contents

Page	Description	When filed and when entered or considered
468	**"Hsu"** Hsu & Cho, *J Biol Chem.* 268(22): 16430-1643, 1993.	Listed on Form PTO-1449 submitted on 04-21-04. Considered by Examiner on 07-07-04
475	**"Kruse"** Kruse *et al. EMBO J.* 11(9): 3237-44, 1992	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-06
483	**"Wilks"** Wilks *et al. Molec. Aspects Med.* 12: 255-265, 1991	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-06
494	**"Ruddle"** Ruddle *et al. J. Exp. Med.* 172: 1193-1200, 1990	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-03
502	**"Ashkenazi"** Ashkenazi *et al. Proc. Natl. Acad. Sci. U.S.A.* 88: 10535-10539, 1991	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-03
507	**"Mori"** Mori *et al. J. Immunol.* 157: 3178-3182, 1996-	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-03
512	**"Piguet"** Piguet *et al. Immunol.* 77: 510-514, 1992	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-03
517	U.S. Patent No. 4,894,439	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-03
534	U.S. Patent No. 4,965,271	Listed on Form PTO-1449 submitted on 03-25-2005. Considered by Examiner on 03-02-03

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Differential Expression and Ligand Binding Properties of Tumor Necrosis Factor Receptor Chimeric Mutants*

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The receptors for tumor necrosis factor (TNF) are represented by two transmembrane proteins, $p55^{TNFR}$ and $p75^{TNFR}$, which are members of a family of cell surface molecules, including the Fas antigen, CD30, CD40, OX40, a Shope fibroma virus protein, and the low affinity p75 nerve growth factor receptor. A common structural feature is a sequence of 40 amino acids that is found in adjacent repeated domains, with 6 cysteine residues in a conserved register. To assess the functional significance of this cysteine-rich domain (CRD), we have constructed chimeric receptors between each TNF receptor and the low affinity nerve growth factor receptor. The chimeric receptor cDNAs were expressed efficiently in COS-1 and 3T3 fibroblasts, as assessed by affinity cross-linking, cell surface biotinylation and immunoprecipitation, and equilibrium binding. Receptors with two CRD of either TNF receptor were incapable of binding TNF, whereas receptors with all four CRD retained the ability to bind TNF with wild type affinity. These results, in conjunction with previous deletion mutation studies, suggest that TNF binding to each receptor requires all four cysteine-rich repeats. Furthermore, analysis of chimeric receptors containing domains of $p55^{TNFR}$ suggests that cytoplasmic sequences directly influence the levels of receptor expression.

Tumor necrosis factor-α (TNF-α)[1] is a cytokine that plays important functions in coordinating immunological and inflammatory responses. TNF is released primarily by activated macrophages to induce changes in cell shape, differentiation, and proliferation and viability of a variety of cell types, including T cells, B cells, polynuclear leucocytes, neutrophils, macrophages, thymocytes, endothelial cells, keratinocytes, glial cells, and fibroblasts (1-3). TNF-α is a 17,000 dalton protein that is capable of forming dimeric and trimeric species. The biologically active form is believed to be a homotrimer (4-6). A related molecule, lymphotoxin or TNF-β, shares 30% identity with TNF-α and elicits similar responses as TNF-α (7).

The mechanisms of signal transduction by TNF are unknown; however, two transmembrane receptors for TNF have been defined by molecular cloning (8-10). The two receptors, p55 and p75, are coexpressed on the surface of most nucleated cells and bind TNF-α and -β with high affinity. The most distinctive structural feature common to the two receptors is the presence in the extracellular domain of four 40-amino acid repeats characterized by 6 cysteines in highly conserved positions. Such repetitive structures have been found in diverse cell surface molecules such as the low affinity p75 NGF receptor, or $p75^{NGFR}$ (11, 12), the Fas antigen (13), T cell antigens OX40 (14) and CD27 (15), B cell antigen CD40 (16), CD30 (17), and the T2 gene of Shope fibroma virus (18). The cysteine-rich domain (CRD) of 40 amino acids defines a family of cell surface molecules that appear to act as potential signaling receptors.

Previous studies have indicated that each TNF receptor is capable of participating in TNF-mediated signal transduction after expression of cloned cDNAs in heterologous cells (19-22). Biological responses include cytotoxicity (23, 24), induction of immediate early genes (25, 26), and induction of scavenging enzymes such as Mn-superoxide dismutase (27). These results imply that either one or both receptors are likely to mediate the pleiotropic effects of TNF in responsive cells. Since the two receptors do not contain an apparent mode of signaling, such as inherent kinase or phosphatase activity, it is assumed receptor-associated molecules acting downstream are required for proper signal transduction.

The repetitive pattern of repeats and the high conservation of cysteine residues in the two TNF receptors imply that the extracellular domains of the two TNF receptors may share similar mechanisms of binding. Deletion analysis of the extracellular region of p55 has demonstrated that the first CRD is necessary for binding, and the fourth CRD was found to be important but not necessary (28). These experiments utilized secreted molecules containing mutant $p55^{TNFR}$ extracellular domains fused to portions of the IgG1 heavy chain, conditions that may not necessarily simulate the native environment of a receptor on the cell surface.

To begin to define the receptor sequences required for TNF binding in a more natural context, we have created a series of chimeric receptors by exchanging cysteine-rich repeated domains with those represented in the low affinity p75 NGF receptor, whose overall structure is closely related to the two TNF receptors (18, 29, 30). We reasoned that unlike deletion mutations that might significantly disrupt receptor structure, these chimeras would be useful for examining which CRD are necessary for TNF binding while preserving the integrity of the extracellular cysteine-rich motif. These chimeric receptors were analyzed for expression, equilibrium binding to ^{125}I-TNF,

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[1] The abbreviations used are: TNF, human tumor necrosis factor-α; TNFR, tumor necrosis factor receptor; NGF, mouse nerve growth factor; NGFR, nerve growth factor receptor; CRD, cysteine-rich domain; bp, base pair(s); BSA, bovine serum albumin; EGS, ethylene glycol *bis* (succinimidylsuccinate); kb, kilobase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

and by affinity cross-linking after transient transfection in COS cells. Our results indicate that TNF binding requires expression of all four cysteine repeats, that substitution of pairs of cysteine repeats from each receptor is not sufficient for binding. Furthermore, binding to chimeric TNF receptors is similar to the equilibrium binding constants that have been measured for wild type TNF receptors.

EXPERIMENTAL PROCEDURES

Construction of Human TNF Receptor Mutants—All recombinant DNA techniques followed standard procedures (31). The plasmid pCMV5A, containing a 1.5-kb cDNA fragment encoding the human $p75^{NGFR}$, plasmid $p55^{TNFR}$ containing a 2.1-kb cDNA fragment encoding human $p55^{TNFR}$ (10), and plasmid $p75^{TNFR}$ containing a 1.5-kb cDNA fragment encoding human $p75^{TNFR}$ (9) were used for the generation of chimeric constructs. All three wild type receptors were cloned into the *Eco*RI site in the polylinker region of the expression vector pCMV5, whose transcription is directed by the cytomegalovirus promoter (32). All constructs utilized selected restriction sites within the receptor cDNAs and are shown schematically in Fig. 1. Chimeric junctions were confirmed by dideoxy DNA sequencing (33) to verify the correct reading frame.

To create a unique restriction site in the $p75^{NGFR}$ cDNA, an *Mlu*I linker encoding Arg-Arg-Val-Ala was inserted at the 400-bp *Nar*I site, generating a modified pCMV5A cDNA vector named pCMV5B. Removal of 71 amino acids from the cytoplasmic domain of $p55^{TNFR}$ was necessary to achieve expression of the receptor (see text for details). A truncated $p55^{TNFR}$ missing 71 amino acids from the cytoplasmic domain was generated after *Eco*RI digestion of plasmid $p55^{TNFR}$, eliminating an 800-bp fragment encoding amino acids 346–417. The truncated $p55^{TNFR}$ plasmid was then religated and named $p55^{TNFR}$tr.

Chimeric constructs are named on the basis of the CRD contributed by the TNF receptor. As described previously, the boundaries of each CRD are defined according to the following pattern: Cys-X_{10-14}-Cys-X_2-Cys-X_2-Cys-X_{9-11}-Cys-X_8-Cys (9–11). Thus, construct (1,2)$p55^{TNFR}$ contains sequences representing the first two CRD (amino acid residues −30–85) from $p55^{TNFR}$ linked to the third and fourth CRD (amino acids 70–222), transmembrane, and cytoplasmic domains of $p75^{NGFR}$. To generate (1,2)$p55^{TNFR}$, plasmid pCMV5B was linearized with *Mlu*I, incubated with the Klenow fragment of *Escherischia coli Pol*I to remove over-hanging ends, and partially digested with *Eco*RI to generate a 5.8-kb cDNA fragment with a blunt end and an *Eco*RI cohesive end. Linearization of plasmid $p55^{TNFR}$ with *Bgl*II followed by Klenow treatment and digestion with *Eco*RI released a 530-bp fragment. The chimeric junction (5′-GTGGA-GATCCGCGTCG-3′) was formed by insertion of the $p55^{TNFR}$ fragment into the modified pCMV5B plasmid to produce ligation of the *Bgl*II and *Mlu*I blunt ends. Because of the modifications made in pCMV5B, 2 residues, Arg and Val, were inserted at the junction.

To create construct (1,2)$p75^{TNFR}$, which represents the first two CRD from $p75^{TNFR}$ (amino acids 1–87) and the last two CRD (amino acids 70–222), transmembrane, and cytoplasmic domains of $p75^{NGFR}$, pCMV5A was digested with *Nar*I followed by Klenow treatment and a partial *Eco*RI digestion. A linearized plasmid containing a 1.1-kb insert with a 5′ blunt *Nar*I end and a 3′ *Eco*RI end ligated to the *Eco*RI site in the polylinker was generated. A 340-bp $p75^{TNFR}$ fragment formed by digesting plasmid $p75^{TNFR}$ with *Eco*RI and *Stu*I was isolated separately and inserted into the pCMV5A vector. The chimeric junction (5′-ACTCAGGCGCCGTGC-3′) occurs between the $p75^{TNFR}$ *Stu*I and $p75^{NGFR}$ *Nar*I blunt ends with no alterations in amino acid sequence.

Chimera (3,4)$p55^{TNFR}$ is composed of the first two CRD of $p75^{NGFR}$ (amino acids −28–70) and the third and fourth CRD (amino acids 86–181), transmembrane and cytoplasmic domains of $p55^{TNFR}$. After linearization of pCMV5B with *Mlu*I, the plasmid was treated with S1 nuclease, followed by digestion with *Bam*HI, liberating a 1.1-kb fragment and retaining 400-bp $p75^{NGFR}$ cDNA sequence. A 3′ 1.7-kb $p55^{TNFR}$ fragment produced from *Bgl*II digestion, S1 nuclease treatment, and *Bam*H1 digestion of the full-length $p55^{TNFR}$ cDNA was inserted into this modified pCMV5B vector. The junction sequence (5′-TCGGCGCGATCTTCT-3′) occurs at the *Mlu*I-*Bgl*II blunt ends and contains an additional arginine residue contributed by the linker modifier in pCMV5B.

To create (3,4)$p55^{TNFR}$tr, pCMV5A was linearized with *Nar*I, digested with S1 nuclease, and partially digested with *Eco*RI, to liberate a 1.1-kb fragment and retain 400-bp 5′ sequences of $p75^{NGFR}$ within the plasmid vector. A 900-bp fragment was isolated from digestion of $p55^{TNFR}$tr with *Bgl*II, followed by S1 nuclease treatment and *Eco*RI digestion, and then introduced into the modified pCMV5A plasmid by DNA ligase. The junction (5′-TCGGCGCGATCTTCT-3′) is formed by ligation of the *Nar*I-*Bgl*II blunt ends.

(3,4)$p75^{TNFR}$ is the product of a ligation between sequences encoding the first two CRD of $p75^{NGFR}$ (amino acids −28–70) and the third and fourth CRD (amino acids 89–240), transmembrane, and cytoplasmic sequences of $p75^{TNFR}$. pCMV5A was digested with *Nar*I, treated with S1 nuclease, and digested with *Kpn*I to generate a 5.1-kb fragment. This was ligated to a 1.16-kb $p75^{TNFR}$ product created by digestion of receptor cDNA with *Kpn*I and *Stu*I. The chimeric linkage (5′-ATGTCGGCCTGCACT-3′) occurs between the *Nar*I and *Stu*I blunt ends. No amino acid changes were generated in this construct.

(1–4)$p55^{TNFR}$ contains all four CRD from $p55^{TNFR}$ (amino acids −28–81) linked to the transmembrane and cytoplasmic sequences from $p75^{NGFR}$ (amino acids 188–400). pCMV5A was digested with *Bsu*36I, treated with Klenow, and partially digested with *Eco*RI to liberate 750 bp of the 5′ $p75^{NGFR}$ sequence and retain 3′ 750-bp sequences encoding the transmembrane and cytoplasmic regions of $p75^{NGFR}$. An 800-bp fragment from $p55^{TNFR}$ generated by *Ban*I digestion, Klenow treatment, and *Eco*RI digestion was then ligated into the modified pCMV5A vector, resulting in a junction without amino acid modification (5′-TCAGGCACTGAGGCA-3′) between the *Bsu*36I and *Ban*I blunt ends.

(1–4)$p75^{TNFR}$ contains all four CRD from $p75^{TNFR}$ (amino acids 1–720) ligated to the transmembrane and cytoplasmic domains of $p75^{NGFR}$ (amino acids 188–400). To create (1–4)$p75^{TNFR}$, *Bsu*36I-digested pCMV5A was used in a ligation reaction to a 790-bp fragment formed by *Ear*I digestion, Klenow treatment, and *Eco*RI digestion of $p75^{TNFR}$. Ligation between the *Bsu*36I and *Ear*I blunt ends resulted in the chimeric junction without any amino acid changes (5′-CTTCCAGTTGGA-3′).

Cells and Transfection—COS-1 monkey kidney cells and 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Transient transfections were carried out by treating a 15-cm dish of COS-1 cells with a calcium phosphate precipitation (34) or lipofectin (Life Technologies, Inc.) mixture containing 15 μg of plasmid DNA. Mock transfections followed similar procedures but lacked plasmid DNA. Cells were harvested 48–72 h after transfection. Stable transfections of 3T3 cells were performed with 1 × 10^6 cells/10-cm dish by cotransfection of a hygromycin resistance plasmid, pHygro, and subsequent maintenance in DMEM supplemented with 0.2 mg/ml hygromycin (Sigma).

Cell Surface Biotinylation, Immunoprecipitation, and Protein Dectection—Procedures for cell surface biotinylation were followed as previously described (35). Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then incubated at 4 °C in the presence of 0.5 mg/ml *N*-hydroxysuccinimide long chain biotin (Pierce Chemical Co.) for 30 min. The biotin solution was removed and replaced with ice-cold serum-free DMEM for 5–10 min. Cells treated with NHS-biotin were then washed twice with cold PBS and lysed in a 1% Nonidet P-40 lysis buffer containing aprotinin (1 mM), leupeptin (1 mM), and phenylmethylsulfonyl fluroide (1 mM) for 20 min on ice.

For immunoprecipitation, lysates were incubated at 4 °C in the presence of the appropriate antibody. A rabbit polyclonal antibody directed against the cytoplasmic region of $p75^{NGFR}$ was kindly provided by Dr. Stu Decker (Parke-Davis). Rabbit polyclonal antibodies directed against the entire extracellular regions of human $p55^{TNFR}$ and $p75^{TNFR}$ receptors were generously supplied by Dr. Link Moldawer (Cornell University Medical College). After a 1-h incubation at 4 °C, Protein A-Sepharose (Sigma) was added and the mixture allowed to incubate an additional hour. The pellet was gently washed once with 1 ml of Buffer I (150 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.2% BSA), three times with Buffer II (150 mM NaCl, 20 mM Tris, pH 3.0, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA), once with Buffer III (500 mM NaCl, 20 mM Tris, pH 8.0, 0.5% Triton X-100, 0.2% BSA), and finally with 50 mM Tris, pH 8.0. Following resuspension in SDS sample buffer and heating for 5 min at 100 °C, proteins were separated by SDS-PAGE on an 8% gel.

Following electrophoresis, immunoprecipitated proteins were transferred as described previously (36) to nitrocellulose paper (Schleicher & Schuell). Nonspecific sites were blocked at room temperature with TGG buffer (PBS, 0.5% Tween-20, 1 M glucose, 10% glycerol) containing 3% BSA, 1% non-fat dry milk powder for 1 h

and were then washed in a PBS-0.5% Tween-20 buffer twice before the blot was treated to a 2-h incubation with 0.3% BSA in TGG containing ^{125}I-streptavidin. After washing in PBS-Tween, proteins were analyzed by autoradiography.

For Western analysis, transfected cells were lysed and immunoprecipitated as above. After transfer to nitrocellulose paper, proteins were probed with anti-p75^{NGFR} polyclonal antibodies followed by ^{125}I-Protein A (Amersham Corp.).

Radiolabeling of TNF-α—TNF-α (Chiron) was labeled with Na^{125}I (Amersham Corp.) and IODO-GEN (Pierce Chemical Co.) as described previously (37). Briefly, 10 μg of human TNF-α was incubated with a prepared film of IODO-GEN (10 μg) at 4 °C in the presence of 1 mCi of carrier-free Na^{125}I. Free iodine was removed by gel filtration on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer containing 0.1% gelatin.

Affinity Cross-linking—Cells were detached by treatment with PBS containing 1 mM EDTA (PBS-EDTA), resuspended at a density of 0.5–2 × 10^7 cells/ml of DMEM, and incubated on a rotating apparatus with 10 nM ^{125}I-TNF at 4 °C. Where indicated, the binding reactions were also performed in the presence of a 100-fold excess of unlabeled human TNF-α. After 1 h, the cross-linking reagent ethylene glycol *bis* (succinimidylsuccinate) (EGS, Pierce Chemical Co.), freshly prepared in dimethyl sulfoxide, was added to a final concentration of 1 mM. After incubation for 40 min at 4 °C, the reaction was quenched by the addition of 20 mM ammonium chloride. Following a 10-min incubation at 4 °C, cells were washed 3 times in ice-cold PBS and then lysed as described above. Insoluble material was removed by centrifugation and cross-linked TNF-receptor complexes were subjected to analysis by SDS-PAGE.

Approximately 5 × 10^6 transfected cells were incubated with ^{125}I-NGF and cross-linked with EDC according to published procedures (11). Cross-linked products were isolated and separated on an 8% polyacrylamide gel and exposed for autoradiography.

TNF-α Binding—Transfected COS-1 cells were detached with PBS-EDTA, resuspended at 3 × 10^6/ml in PBS containing 1 mg/ml BSA and glucose, and incubated with various concentrations of ^{125}I-TNF-α in the absence or presence of a 100-fold excess of cold TNF-α for 2 h at 4 °C. Free ^{125}I-TNF was separated from receptor-bound ^{125}I-TNF by centrifugation (15,000 rpm) through a 1-ml calf serum cushion in 1.5-ml microcentrifuge tubes. Total binding was then assessed in a scintillation counter. Nonspecific binding was subtracted and Scatchard analysis performed on data. Binding experiments were performed in duplicate.

RESULTS

Experimental Strategy—The p55 and p75 TNF receptors are distinguished by a cysteine-rich 40-amino acid motif that is repeated four times in their extracellular regions. To investigate the importance of the CRD in each TNF receptor in ligand binding, we constructed chimeric receptor molecules using cDNAs encoding the 55- and 75-kDa human TNF receptors and the 75-kDa low affinity human NGF receptor. We selected restriction sites in the full-length cDNAs that were placed near the CRD boundaries as defined earlier (9–11) and used restriction digest fragments to construct most of the mutant receptors, which are named according to the CRD contributed by the TNF receptors (Fig. 1).

Three different sets of chimeric cDNA molecules were constructed: 1) sequences encoding the first two CRD from either the p55 or p75 TNF receptor were fused to cDNA encoding the third and fourth CRD, transmembrane domain, and cytoplasmic region of p75^{NGFR} (constructs (1,2)p55 and (1,2)p75); 2) sequences encoding the first two CRD from p75^{NGFR} were fused to sequences encoding the last two CRD, transmembrane, and cytoplasmic domains from each TNF receptor (constructs (3,4)p55, (3,4)p55tr, and (3,4)p75); and finally, 3) all four CRD from both TNF receptors were each linked to the sequences encoding the transmembrane and cytoplasmic domains of p75^{NGFR} (constructs (1–4)p55 and (1–4)p75) (Fig. 1). The cDNA sequences for the chimeric receptors were inserted into pCMV5 (32), which contains a cytomegalovirus promoter and SV40 origin of replication and is capable of high levels of expression after transfection into monkey kidney COS-1 cells.

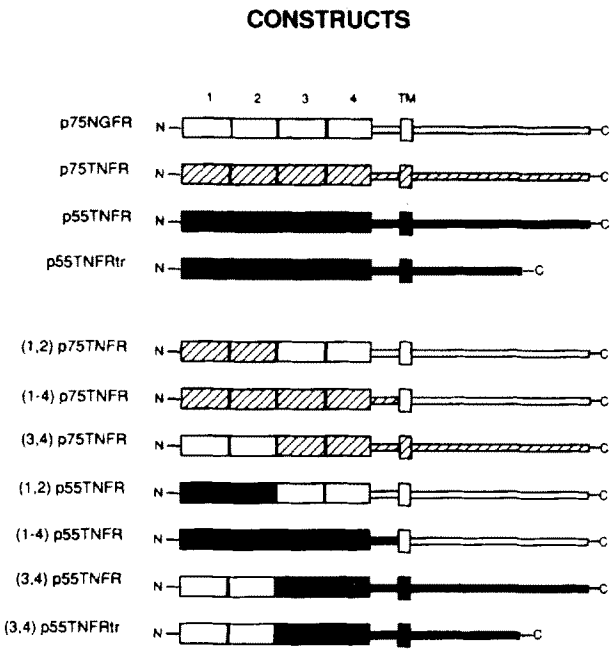


FIG. 1. **Schematic representation of TNF and NGF chimeric receptors.** Chimeric receptors are named based on the CRD contributed by the TNF receptor. Boundaries of each CRD were determined previously (9–11). The reading frame for each chimera was verified by DNA sequencing. *Blank regions* represent p75^{NGFR}; *cross-hatched regions* represent p75^{TNFR}; *stippled regions* represent p55^{TNFR}.

Expression of Chimeric TNF Receptors—To confirm that chimeric receptors are being efficiently expressed, Western blot analysis was performed. COS cells transfected with the chimeric constructs containing the cDNA encoding the intracellular domains of p75^{NGFR} were lysed and immunoprecipitated with rabbit polyclonal antibodies recognizing the cytoplasmic tail of p75^{NGFR}. The immunoprecipitated lysates were transferred to nitrocellulose and incubated with the same antibody, followed by ^{125}I-Protein A (Fig. 2, *A* and *B*). All receptor cDNAs screened in this manner expressed a detectable protein. Protein mobility varied accordingly from either protein truncation or changes in glycosylation.

Detection of chimeric receptors by immunoblot analysis does not necessarily indicate whether these receptors are expressed at the cell surface. To verify that these receptors are expressed at the cell surface, a cell surface biotinylation procedure was utilized. Cells were incubated with biotin at 4 °C to prevent internalization and ensure that only surface proteins were labeled. Cell lysates were then subjected to immunoprecipitation with antibodies directed against the extracellular domains of the p55 or p75 TNF receptor. Chimeric receptors containing cytoplasmic sequences of p75^{NGFR} were also analyzed using antibodies directed against the cytoplasmic domain of p75^{NGFR}. Following electroblot transfer, the biotinylated proteins were visualized after incubation with ^{125}I-streptavidin followed by autoradiography (Fig. 3). All of the chimeric receptor molecules are expressed on the cell surface. It should be noted that chimeras (1,2)p55 and (1,2)p75 are efficiently immunoprecipitated by antibodies to their NGF receptor domains as well as antibodies directed against the TNF receptor domains (data not shown).

A third approach confirmed the appearance of appropriate

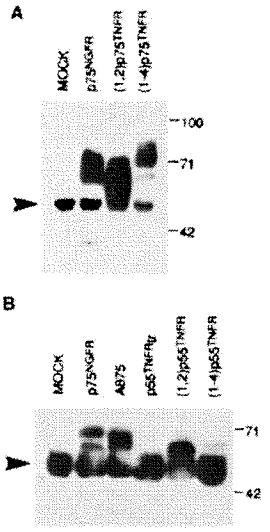
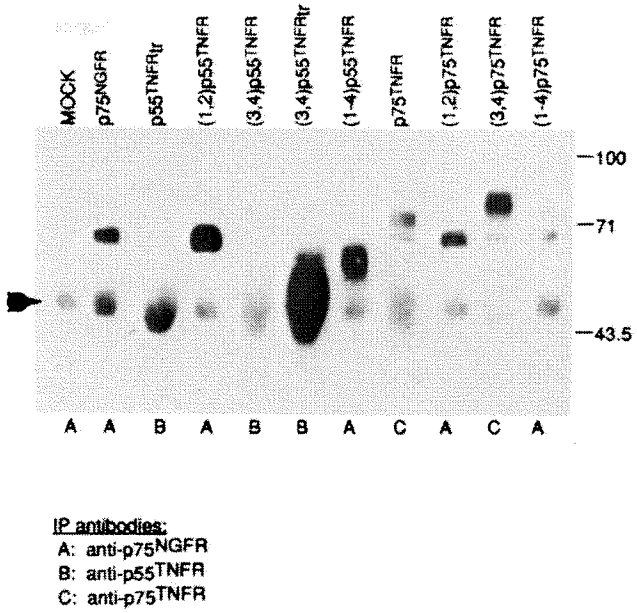


FIG. 2. **Immunoblot analysis of chimera expression.** *A*, COS-1 cells were transfected with cDNA encoding $p75^{NGFR}$, $(1,2)p75^{TNFR}$, and $(1–4)p75^{TNFR}$. Cell lysates were incubated with an anti-$p75^{NGFR}$ polyclonal antibody directed against the cytoplasmic domain of $p75^{NGFR}$. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose paper, and incubated with the same antibody followed by ^{125}I-Protein A. *B*, COS-1 cells were transfected with cDNA encoding $p75^{NGFR}$, $p55^{TNFR}$tr, $(1,2)p55^{TNFR}$, and $(1–4)p55^{TNFR}$. Immunoprecipitation and detection were as described above. Neither mock transfectant lysates (*MOCK*) nor $p55^{TNFR}$tr lysates contain protein recognized by anti-$p75^{NGFR}$ antibodies. A875 is a cell line expressing the low affinity p75 NGF receptor. Mock transfections were performed in the absence of plasmid DNA. *Solid arrowheads* denote the IgG heavy chain recognized by ^{125}I-Protein A.



IP antibodies:
A: anti-$p75^{NGFR}$
B: anti-$p55^{TNFR}$
C: anti-$p75^{TNFR}$

FIG. 3. **Cell surface expression of chimeric receptors demonstrated by cell surface biotinylation and immunoprecipitation.** COS-1 cells were transfected with the cDNA listed *above each lane*. Receptor proteins were immunoprecipitated after *N*-hydroxysuccinimide long chain biotin labeling of cell surface proteins using the rabbit polyclonal antibody, indicated by a *letter* at the *base* of *each lane*. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with ^{125}I-streptavidin. *Solid arrowheads* denote nonspecific binding of the IgG heavy chain to ^{125}I-streptavidin (35). $p55^{TNFR}$tr and $(3,4)p55^{TNFR}$tr comigrate closely with the IgG heavy chain.

cell surface expression of several chimeric receptors ((1,2)p75, (3,4)p75, (1–4)p75, (1–4)p55) after gene transfer. Receptors expressed on the surface of 3T3 cells stably transfected with chimeric cDNA were detected using monoclonal antibodies against epitopes in the extracellular domains of the NGF receptor and TNF receptors in rosetting experiments (data not shown). Recognition of these receptors by monoclonal antibodies indicates that native epitopes are exposed and suggests receptor structure is maintained.

Affinity Cross-linking of Chimeric Receptors—Affinity cross-linking has previously been employed to characterize expression of TNF receptors on the cell surface (4, 39, 40). We therefore performed cross-linking experiments with ^{125}I-TNF-α to determine whether any of the chimeric receptors are capable of TNF binding. Transfected COS cells were incubated with radiolabeled TNF-α for 1 h at 4 °C. Incubations were carried out in the presence or absence of an excess of unlabeled TNF-α to demonstrated binding specificity. Ligand-bound complexes were then chemically cross-linked with EGS (Pierce Chemical Co.) (see "Experimental Procedures"). EGS was chosen from a panel of cross-linking agents, which included disuccinimidyl suberate, dimethylpimelimidate, *bis* [2-(succinimidooxy-carbonyloxy)ethyl]sulfone, and *bis* (sulfosuccinimidyl) suberate, to be most efficient at affinity cross-linking of TNF receptors.

When a chimeric receptor containing all four CRD of p75 was tested in COS cells, cross-linking to ^{125}I-TNF could easily be observed (Fig. 4*A*, *lane 7*). Similar results were found with the full-length p75 TNF receptor (Fig. 4*A*, *lane 3*). However, cross-linking was not observed for the chimeric receptors containing the first two CRD or the third and fourth CRD of the p75 TNF receptor (Fig. 4*A*, *lanes 5* and *9*), even though both chimeric receptors were expressed on the cell surface (Fig. 3).

Analogous results were obtained with the p55 chimeric receptors. Cross-linking was observed with a receptor containing all four extracellular CRD from p55 (Fig. 4*B*, *lane 7*), whereas chimeras containing the first two CRD (Fig. 4*B*, *lane 5*) or third and fourth CRD (data not shown) did not display cross-linking. These results indicate that the first two and second two CRD of the p55 and p75 TNF receptors are necessary, but not sufficient, for binding TNF.

Stably transfected 3T3 cell lines expressing these TNF receptor cDNAs were generated after transfection with pCMV expression plasmids. Positive clones were isolated after hygromycin selection and screened for cell surface expression by rosetting with monoclonal antibodies. Affinity cross-linking of these cell lines to ^{125}I-TNF verified the results obtained by the transient COS expression system (data not shown).

NGF Binding—The stably transfected cell lines were used to assess the ability of the TNF chimeric receptors to bind NGF in affinity cross-linking studies using ^{125}I-NGF. Linker insertion mutagenesis of the $p75^{NGFR}$ demonstrated that the third and fourth CRD contribute to NGF binding (41). Chimeric receptors that bind TNF, such as (1–4)p75, did not bind to ^{125}I-NGF, indicating that the two receptor systems do not cross-react. Also, a chimeric receptor that retains the third and fourth cysteine repeats of $p75^{NGFR}$ did not display cross-linking to ^{125}I-NGF (Fig. 5). These results demonstrate that structural features of all four cysteine repeats must be present for appropriate binding of each receptor to TNF and NGF.

$p55^{TNFR}$ Expression—High level expression of the full-length p55 TNF receptor has been difficult to achieve after gene transfection experiments, leading investigators to sug-

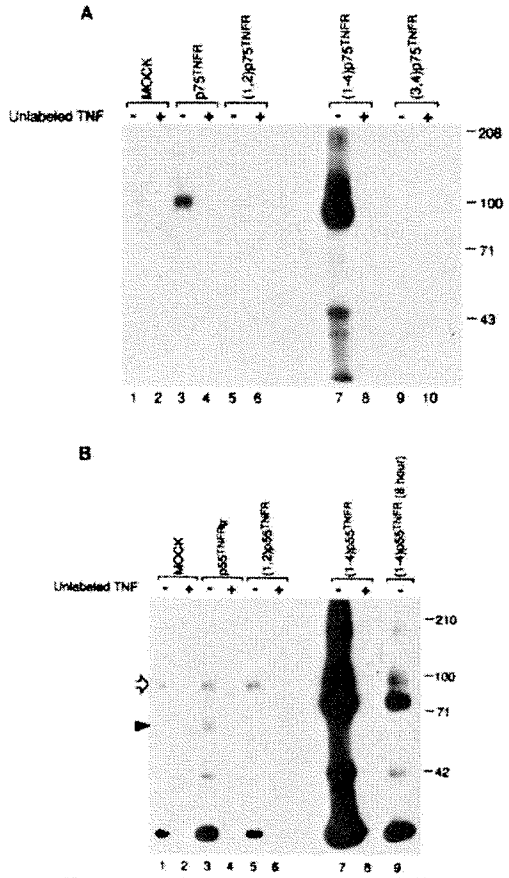


FIG. 4. **^{125}I-TNF affinity cross-linking of COS-1 cells expressing TNF receptors and chimeric receptors.** Approximately 3×10^6 cells were incubated with ^{125}I-TNF, in the absence (-) or presence (+) of a 100-fold excess of unlabeled TNF and incubated with EGS. Cross-linked products were analyzed by 8% polyacrylamide gel electrophoresis. Cross-linked ^{125}I-TNF dimers and trimers are detected at 30 and 45 kDa, respectively. *A*, COS-1 cells were either mock-transfected (*MOCK*) (*lanes 1* and *2*) or transfected with cDNA encoding the full-length p75^{TNFR} (*lanes 3* and *4*), (1,2)p75^{TNFR} (*lanes 5* and *6*), (1–4)p75^{TNFR} (*lanes 7* and *8*), or (3,4)p75^{TNFR} (*lanes 9* and *10*). *B*, COS-1 cells were mock-transfected (*lanes 1* and *2*) or transfected with cDNA encoding the truncated p55 TNF receptor p55^{TNFR}tr (*lanes 3* and *4*) or chimeric receptors (1,2)p55^{TNFR} (*lanes 5* and *6*) and (1–4)p55^{TNFR} (*lanes 7* and *8*). *Lane 9* is a shorter exposure of *lane 7*. *Solid arrowheads* denote the cross-linked products for the truncated p55 TNF receptor. *Open arrowheads* denote endogenous p75 TNF receptors expressed on COS-1 cells.

gest that sequences in the 5′-untranslated region and/or cytoplasmic regions may be responsible for the reduced translation efficiency (19, 21, 42). Interestingly, Loetscher *et al.* (10) achieved much higher levels by removing the carboxyl-terminal cytoplasmic domain of p55. Therefore, to achieve higher levels of p55, we eliminated sequences encoding 71 amino acids from the carboxyl terminus by digestion with *Eco*RI (Fig. 1).

The truncated p55 receptor (Fig. 4*B*, *lane 3*) was detected more easily than the full-length p55 receptor but was visualized after affinity cross-linking only after long exposure times, during which time endogenous p75 receptors become evident (Fig. 4*B*). It should be noted that the p55 TNF receptor chimeras (1–4)p55 and (1,2)p55 are also highly expressed as assessed by cross-linking (Fig. 4*B*) and cell surface biotinylation (Fig. 3). Interestingly, both of these chimeric receptors contain the native 5′-untranslated sequences that have been

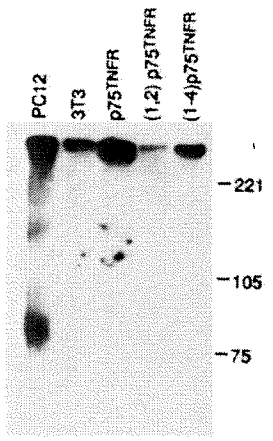


FIG. 5. **^{125}I-NGF affinity cross-linking of TNF chimeric receptors.** Approximately 6×10^6 cells were incubated with ^{125}I-NGF and cross-linked with EDC. PC12 cells express the low affinity p75 NGF receptor. 3T3 cells or 3T3 cells stably transfected with cDNA encoding p75^{TNFR}, (1,2)p75^{TNFR}, or (1–4)p75^{TNFR} do not display any detectable cross-linking with ^{125}I-NGF.

suspected to decrease expression (19, 21, 42). The expression data suggest that the sequences responsible for decreased expression lie in the cytoplasmic region of the receptor and that truncation of the cytoplasmic sequences enhances expression.

To test this possibility, we compared the expression of chimeric receptors that differed in carboxyl-terminal information. Expression of construct (3,4)p55, which contains the intact cytoplasmic sequences of p55, was relatively low. Removal of 71 amino acids from the cytoplasmic sequence consistently resulted in high level expression for these constructs, implying that this intracellular sequence affects p55 TNF receptor expression (Fig. 3, *lanes 5* and *6*).

Equilibrium Binding of ^{125}I-TNF to Chimeric Receptors—To characterize the TNF binding properties of the chimeric receptors, Scatchard analysis was performed on binding data from transfected cells incubated with decreasing concentrations of radioiodinated human TNF-α. COS-1 cells have a K_d of 2 nM, which reflects the binding of endogenous TNF receptors. As examined by steady state binding experiments, COS-1 cells express endogenous receptors but at very low levels (8, 10). COS cells expressing the p75 TNF receptor bind with a K_d of 6 nM (Fig. 6), with approximately 2×10^5 binding sites/cell. These measurements are comparable with previous findings.

An increase in the number of binding sites was not detected when chimeric receptors containing only two of the four TNF receptor CRD were expressed, consistent with the inability of these receptors to bind TNF (Table I). A dramatic increase in binding sites (40,000–300,000) was observed with receptors containing four CRD, either from the p55 or p75 TNF receptor. Consistent with the cross-linking data, the number of sites was nearly two orders of magnitude higher than endogenous levels. Equilibrium dissociation constants measured for these receptors (2–6 nM) was very similar to wild type p75 and truncated p55 TNF receptors. These binding values again indicate that all four CRD appear to be sufficient for TNF binding and that the affinity of TNF binding to the extracellular CRD structure was not affected in the context of these chimeric constructs.

DISCUSSION

The striking conservation of the cysteine-rich motif found in four extracellular domains of the TNF receptors suggests

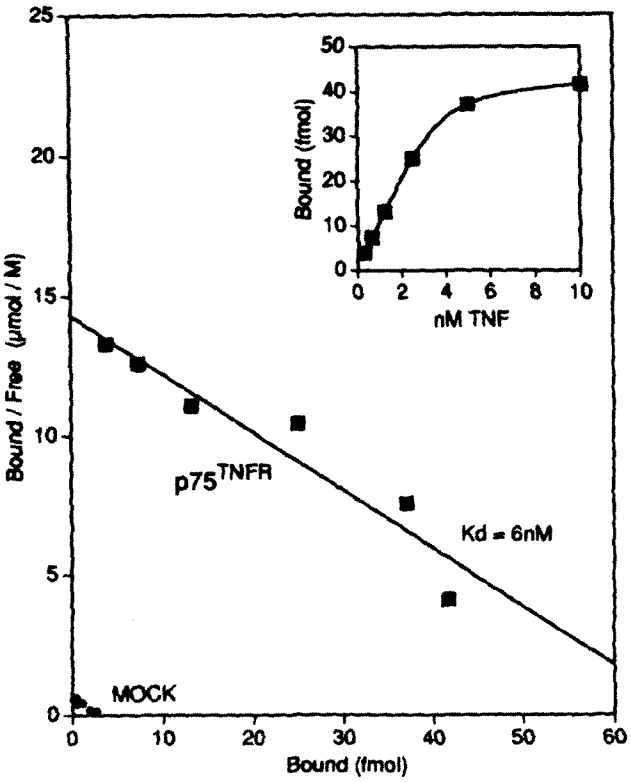


FIG. 6. **TNF binding and Scatchard analysis of COS-1 cells transfected with $p75^{TNFR}$.** COS-1 cells transfected with cDNA encoding the full-length $p75^{TNFR}$ were incubated with increasing concentrations of ^{125}I-TNF for 2 h at 4 °C and assayed for binding (*inset*), as described under "Experimental Procedures." Scatchard analysis was performed on the binding data. ■, cells transfected with plasmid $p75^{TNFR}$; ●, cells that were mock-transfected.

TABLE I

Summary of TNF binding sites and dissociation constants in wild type and chimeric TNF receptors transiently expressed in COS-1 cells

DNA construct	Affinity constant (K_d)	Sites/cell
	nM	
MOCK[a]	2	5.8×10^3
$p75^{NGFR}$	7	6.4×10^3
$p55^{TNFR}$	1.3	3×10^3
$p55^{TNFR}{}_{tr}$	2	4.3×10^4
(1,2)$p55^{TNFR}$	2	2.7×10^3
(3,4)$p55^{TNFR}$	2	2.3×10^3
(1–4)$p55^{TNFR}$	2	1.7×10^6
$p75^{TNFR}$	6	1.5×10^5
(1,2)$p75^{TNFR}$	1	2.6×10^3
(3,4)$p75^{TNFR}$	1.5	1.3×10^3
(1–4)$p75^{TNFR}$	5	2.54×10^5

[a] MOCK, mock transfectant lysates.

that each CRD may exist as a basic unit of structure, important in receptor conformation and ligand binding. We have created chimeric receptors between the two TNF receptors and $p75^{NGFR}$ to generate transmembrane receptor molecules whose individual CRD have been selectively exchanged but whose conformation is likely to be conserved. Mutant receptors were expressed after transfection into cultured cells, as assayed by immunoreactivity with specific antibodies either to the TNF receptor or NGF receptor. Moreover, they are transported to the cell surface, as determined by immunoprecipitation of biotinylated cell surface proteins.

Ligand binding studies demonstrate that all four CRD from either TNF receptor are required for specific ligand binding and that two adjacent CRD from either TNFR are not sufficient. Affinity cross-linking was observed with chimeric receptors containing all four CRD, whereas cross-linking of ^{125}I-TNF was not detected in cells transfected with receptor constructs containing only two of the four TNF receptor CRD, either the first two or the third and fourth. By Scatchard analysis, chimeric receptors with a complete series of CRD bind TNF-α with an affinity similar to wild type receptors, indicating that an intact CRD structure is necessary for appropriate TNF binding.

A previous mutagenesis study of the p55 receptor utilized extracellular domains fused to the hinge and Fc portion of IgG1 heavy chain to demonstrate that deletion of the first or fourth CRD results in complete elimination of binding or diminished binding, respectively, suggesting a contribution from multiple CRD for ligand binding (28). The data presented here address both TNF receptors and demonstrate that each domain cannot be exchanged with analagous domains from other receptors and retain functional binding properties. Adjacent CRD, when transplanted to the p75 NGF receptor, do not bind TNF, presumably due to the lack of the missing TNF receptor CRD or possibly due to the different molecular environment. For example, the $p75^{NGFR}$ CRD structure is strongly negatively charged, while the TNF receptor CRD sequence is positively charged. However, since binding was not observed in the chimeras containing two TNF receptor CRD by affinity cross-linking and steady state binding, we conclude that additional sequences from other CRD are necessary for wild type TNF binding. These results imply that elements of all four CRD in $p55^{TNFR}$ and $p75^{TNFR}$ are required for interactions with TNF. The recently solved crystal structure of the extracellular domain of $p55^{TNFR}$ indicates that the four repeats form an elongated ladder-like structure in which multiple contacts exist between TNF-α and the receptor (38). Our findings that all four cysteine-rich repeats are required for binding is consistent with this model.

The inability of some of the receptors to bind TNF could be due to a gross conformational alteration of the receptor structure. Several experimental findings argue against this possibility. The receptors are efficiently recognized by antibodies produced against extracellular portions of the TNF and NGF receptors, including antibodies directed against specific epitopes. Antibody recognition of mutant receptors was demonstrated in several assays, including immunoprecipitation with polyclonal antibodies and cell surface rosetting with monoclonal antibodies, suggesting correct folding and processing. It is interesting to note that the polyclonal antisera were ineffective at detecting p55 and p75 in Western blot experiments but efficiently immunoprecipitated the receptors. This indicates that native epitopes are recognized by the antibodies in a relatively nondenatured state and suggests that the structure of the chimeric receptors is preserved. The inability of TNF to bind to the receptors containing two of four CRD more likely is due to changes in amino acid sequence or overall charge than to gross conformational changes.

Chimeric receptor (1,2)p55 required the insertion of a linker to create a construct with a novel restriction site. This insertion resulted in the addition of two residues at the chimeric junction, which could potentially affect protein folding. The (1,2)p55 receptor molecule, however, is recognized by antibodies directed against the extracellular domain of p55.

Analysis of NGF binding to these chimeras demonstrated that disruption of the native structure of the receptor resulted in loss of ligand binding. These results are consistent with previous mutagenesis studies of $p75^{NGFR}$ (41, 43, 44), which

suggested that multiple CRD of this molecule contributed to NGF binding. Deletion analysis as well as linker insertion studies demonstrated that mutations in the third and fourth CRD have a negative effect upon NGF binding. Interestingly, cyclic permutations of the first CRD after the fourth CRD yield an alternative receptor that did not display binding to NGF (44), suggesting that the order and sequence of the CRD are also essential to binding.

Previous findings indicated that high level expression of the full-length cDNA for the p55 receptor was difficult to obtain after transfection (19, 21, 42). One hypothesis is that sequences in the 5′ noncoding region act negatively upon expression of $p55^{TNFR}$. We have tested this possibility by analyzing the expression of cDNAs that retain these 5′ sequences. Chimeric receptors (1,2)p55 and (1–4)p55 contain the 5′-untranslated sequences and are expressed at high levels, suggesting that these sequences alone cannot account for the lack of expression of full-length p55.

Instead, cytoplasmic sequences may account for decreased expression, since carboxyl-terminal truncation of p55 results in higher expression. This alternative was explored in further studies using chimeric receptors. Chimera (3,4)p55, which lacks the untranslated 5′ p55 sequences but retains the entire cytoplasmic sequence of wild type p55, was not expressed at detectable levels at the cell surface. When 213 base pairs (71 amino acids) from the cytoplasmic sequence of the chimera was removed, receptor expression levels were significantly higher. This result suggests that the cytoplasmic sequences of the p55 TNF receptor influence expression independent of the 5′-untranslated sequences. The significance of the cytoplasmic sequence and the mechanism by which it limits receptor expression are unknown but may involve alteration of mRNA stability or post-translational modification.

Alternatively, high levels of expression of the sequence under transfection conditions may have deleterious effects on cells. The deleted sequences contain a region found to have limited homology to cytoplasmic sequences in the Fas antigen, a molecule associated with apoptotis (13). In addition, functional analysis of this cytoplasmic amino acid sequence has demonstrated a possible role in cytotoxicity, since its deletion abolishes the cytocidal effect of TNF in a number of cell lines (19, 42). Expression of the cytoplasmic sequence at higher than normal levels may trigger cytocidal mechanisms and result in cell death. The influence and function of the p55 cytoplasmic sequences are clearly not well defined and will require a more detailed cell biological analysis.

These mutant receptors will be useful in studies concerning TNF binding and events downstream of receptor-ligand interaction. Receptor clustering has been described by others (4, 45, 46) using TNF and anti-$p55^{TNFR}$ antibodies. Chimeric receptors expressed in TNF-responsive cell lines may alter the traditional response to TNF either by associating with or by sequestering ligand from endogenous signaling receptors or by acting in a dominant negative fashion.

Neither TNF receptor displays amino acid sequence homology to other growth factor receptors that function as protein kinases. TNF receptor-mediated signaling will likely require association with auxillary molecules possessing signaling properties. The mechanism by which ligand binding to receptors with multiple cysteine-rich repeats is translated through the membrane to activate intracellular pathways is a central issue that will likely be solved by an increased understanding of receptor structure and function.

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Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement

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Interleukin-4 (IL-4) represents a prototypic lymphokine (for a recent review see Paul, 1991). It promotes differentiation of B-cells and the proliferation of T- and B-cell, and other cell types of the lymphoid system. An antagonist of human IL-4 was discovered during the studies presented here after Tyr124 of the recombinant protein had been substituted by an aspartic acid residue. This IL-4 variant, Y124D, bound with high affinity to the IL-4 receptor (K_D = 310 pM), but retained no detectable proliferative activity for T-cells and inhibited IL-4-dependent T-cell proliferation competitively (K_i = 620 pM). The loss of efficacy in variant Y124D was estimated to be >100-fold on the basis of a weak partial agonist activity for the very sensitive induction of CD23 positive B-cells. The subsitution of Tyr124 by either phenylalanine, histidine, asparagine, lysine or glycine resulted in partial agonist variants with unaltered receptor binding affinity and relatively small deficiencies in efficacy. These results demonstrate that high affinity binding and signal generation can be uncoupled efficiently in a ligand of a receptor belonging to the recently identified hematopoietin receptor family. In addition we show for the first time, that a powerful antagonist acting on the IL-4 receptor system can be derived from the IL-4 protein.

Key words: drug design/partial agonists/receptor signalling

Introduction

Interleukin-4 (IL-4) represents a typical immunoregulatory lymphokine (for review see Paul and Ohara, 1987; Finkelman *et al.*, 1990; Paul, 1991). It is produced mainly by activated T-cells and mast cells and has a wide range of biological activities on various cell types of the lymphoid system. B-cells are stimulated *in vitro* to express class II major histocompatibility complex (MHC II) molecules, the IgE low affinity receptor (FcεRII, CD23) and immunoglobulins class E and G1. DNA synthesis is induced in activated B-cells, as well as in mature T-cells, foetal thymocytes, mast cells and others.

Mice treated with neutralizing antibodies directed against IL-4 or against the IL-4 receptor (Finkelman *et al.*, 1990; Urban,Jr *et al.*, 1991), as well as mice bearing an inactivated IL-4 gene (Kühn *et al.*, 1991), are unable to produce IgE and show reduced serum levels of IgG1 after challenge with the relevant stimuli. This indicates a physiological role of IL-4 at least in the regulation of IgE levels, most likely by acting as an isotype switching factor. Consequently, IL-4 may exert a pathophysiological role in the generation of disease states, as for example hyper-IgE syndrome or IgE-mediated allergic conditions (see Finkelman *et al.*, 1990; Tepper *et al.*, 1990).

IL-4 function is mediated by its binding to plasma membrane receptors occurring at the relatively small numbers of 150-2500 molecules per target cell (Cabrillat *et al.*, 1987; Park *et al.*, 1987). A single dissociation constant of ~100 pM has been determined for this interaction. The IL-4 receptor probably comprises only a single polypeptide chain of ~800 amino acid residues as suggested by chemical crosslinking experiments using ^{125}I labelled IL-4 (Mosley *et al.*, 1989; Galizzi *et al.*, 1990; Harada *et al.*, 1990; Idzerda *et al.*, 1990). Binding to IL-4 *in vitro* is not influenced by structural elements apart from the extracellular portion of the receptor, since similar dissociation constants were found for association with IL-4 with the entire receptor and only the ligand binding domain in the case of both the mouse and the human protein (Maliszewski *et al.*, 1990; Garrone *et al.*, 1991). At present it is unclear if a second protein aggregates with the receptor after IL-4-induced activation. The IL-4 receptor is evolutionarily related to the large family of hematopoietin receptors, including receptors (or receptor subunits) for interleukins-2, 3, 4, 5, 6 and 7, for GM- and G-CSF and also for erythropoietin, growth hormone and prolactine (Bazan, 1990a,b; Cosman *et al.*, 1990). All these proteins show particular homologies in the amino acid sequence of their extracellular ligand binding domains.

NMR studies revealed a three-dimensional structure of human IL-4 comprising a four-helix bundle motif with an up-up-down-down connectivity (Redfield *et al.*, 1991) similar to both human and porcine growth hormone (Abdel-Meguid *et al.*, 1987; De Vos *et al.*, 1992). Other ligands binding to members of the haematopoietin receptor family also appear to be helically organized proteins (see Bazan, 1990a,b). The crystal structure of human IL-2 shows a four-helix bundle (Brandhuber *et al.*, 1987) albeit with a connectivity different from IL-4. The amino acid sequences of these ligands display no clear homologies. A puzzling similarity, however, is apparent in the pattern of amino acid residues forming one surface of a putative amphipathic helix at the C-terminus of these proteins (Sanderson *et al.*, 1988; see Bazan, 1990b). Sequence positions involved in receptor binding have been identified in several ligands of the haematopoietin receptor family, e.g. in IL-2 (Collins *et al.*, 1988; Weigel *et al.*, 1989; Zurawski and Zurawski, 1989), growth hormone (Cunningham and Wells, 1991; Cunningham *et al.*, 1991), GM-CSF and IL-5 (Shanafelt *et al.*, 1991). Amino acid residues involved in signal generation, however, have been more difficult to identify. In order to address this issue, contributions of particular

amino acids to receptor affinity and biological activity, respectively, have to be distinguishable. As yet, uncoupling of receptor binding from biological effects has been observed to some extent only in the case of certain mutant derivatives of both human and murine IL-2 (Liang *et al.*, 1988; Zurawski *et al.*, 1990).

Recently we have generated a set of mutant variants of human IL-4 with single substitutions at the positions of all cysteinyl and aromatic residues (Kruse *et al.*, 1991). One of these variants, Y124D, was completely inactive even at micromolar concentrations during a T-cell proliferation assay. Subsequently, we realized that variant Y124D efficiently inhibits IL-4 induced T-cell proliferation. This promoted the generation of IL-4 variants in which Tyr124 was substituted by residues differing in size, polarity or charge, respectively. The receptor binding affinity of these isolated mutant proteins was determined, as well as their biological activities in two *in vitro* assays differing in sensitivity by nearly two orders of magnitude. The results of these experiments indicated that the nature of the side chain at position 124 of human IL-4 dramatically affects signal generation and biological activity, while the influence on receptor binding is marginal.

The demonstration of the feasibility to obtain an efficient antagonist of the IL-4 receptor system has several important implications. First, inhibitory IL-4 variants represent potential drugs helpful for example in the treatment of IgE-mediated diseases (see e.g. Finkelman *et al.*, 1990; Paul, 1991) similar to therapeutic monoclonal antibodies against IL-4 or to the soluble extracellular IL-4 receptor domain (Maliszewski *et al.*, 1990; Garrone *et al.*, 1991). Secondly, antagonistic ligands may prove valuable tools for the investigation of IL-4 receptor-mediated signal transducing mechanisms. Thirdly, considering the homologies and similarities mentioned above, it is an intriguing possibility that similar antagonistic variants will be found for other ligands of this receptor family (see also Zurawski *et al.*, 1990).

Results

Reduced efficacy of IL-4 variants in T-cell proliferation

Recombinant human IL-4 promoted DNA synthesis in prestimulated T-cells with a half-maximal response at a concentration (EC_{50}) of 230 pM (Figure 1). Substitution of Tyr124 by aspartic acid near the C-terminus of the 129-residue Il-4 resulted in the mutant protein Y124D, which had no measurable activity in the T-cell proliferation assay (see also Kuruse *et al.*, 1991). Substitution of Tyr124 by a series of other amino acid residues caused less pronounced alterations. As the mutant protein Y124F had the same bioactivity as wild type IL-4 (see Table I for statistical evaluations), the hydroxyl group of Tyr124 is not essential. His124 caused a small but significant reduction of the maximal response. Asparagine, lysine or glycine at this position reduced the potency to 29–13% of wild type IL-4. A comparison of mutant proteins Y124N and Y124D clearly demonstrated that the introduction of a negative charge caused the most dramatic effect on IL-4 activity. Remarkably, the half-maximal response of the residual activity (EC_{50}) of the mutant proteins remained within the same concentration range (130–230 pM) as found for wild type IL-4. This is a typical characteristic of partial agonists for which binding and signal generation (efficacy) are

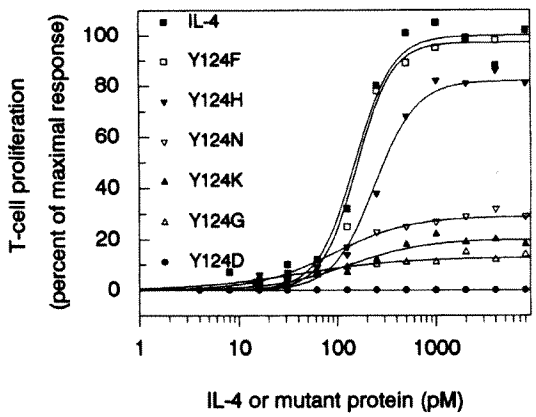

Fig. 1. Proliferation of prestimulated T-cells (PHA blasts) in response to increasing concentrations of IL-4 (■) and mutant proteins Y124F (□), Y124H (▼), Y124N (▽), Y124K (▲), Y124G (△) and Y124D (●).

partially uncoupled (Ruffolo,Jr, 1982; Black, 1989). The unaltered EC_{50} values for the variants further suggest that the response is limited by receptor occupancy (Ruffolo,Jr, 1982). Mutant proteins affected in receptor binding (see e.g. Collins *et al.*, 1988; Weigel *et al.*, 1989; Zurawski and Zurawski, 1989; Cunningham and Wells, 1991; Cunningham *et al.*, 1991; Shanafelt *et al.*, 1991) show a different behaviour such that they produce a maximal response similar to the wild type if applied in sufficient concentrations.

Partial agonism of IL-4 variants in B-cell differentiation

Compared with the T-cell proliferation assay, much lower concentrations of IL-4 were sufficient to stimulate B-cells for the induction of CD23 (FcεRII), the low affinity IgE receptor (Kikutani *et al.*, 1986; DeFrance *et al.*, 1987). After stimulation, the number of CD23 positive cells increased with EC_{50} of ~0.4 pM for IL-4 (Figure 2A) (see also Solari *et al.*, 1989). In this very sensitive assay, mutant protein Y124D behaved as a partial agonist. The number of B-cells that were stimulated by Y124D to express CD23 was up to 45% of that found for wild type IL-4, with an EC_{50} of 210 pM. Very high IL-4 concentrations did not increase the number of CD23 positive cells further, but stimulated higher mean CD23 expression in the responding cells as also determined by FACS analysis. The mean EC_{50} was 5 pM IL-4 in different experiments (see Table I). Mutant protein Y124D induced a maximal response of 8% compared with wild type IL-4 (Figure 2B). Half-maximal response occurred at 310 pM Y124D. These results clearly established that the mutant protein Y124D has a very weak partial agonist activity that was detectable only in the sensitive B-cell assay. The effective concentration for the half-maximal response of 210 pM and 310 pM, however, was in the range of the EC_{50} found for IL-4 and the other mutant proteins in the T-cell system.

Variant Y124G induced a maximal number of CD23 positive B-cells (99%) and a maximal mean CD23 content (77%) similar to IL-4 (= 100%). The EC_{50} for these responses, however, were found to be increased several-fold to ~5 and 40 pM, respectively (Figures 2A and B, see also Table I). Seemingly, these are the properties of a variant defective in receptor binding. However, Y124G shows normal receptor affinity during competitive radioligand binding to B-lymphoma cells and normal EC_{50} during the

Table I. Effective concentration (EC_{50}) and maximal response (R_{max}) of IL-4 and Il-4 variants evaluated from T- and B-cell assays

Protein	T-cell proliferation		B-cell (spleen)			
			Mean CD23 content		Number of CD23 positive cells	
	EC_{50} (pM)	R_{max} (%)	EC_{50} (pM)	R_{max} (%)	EC_{50} (pM)	R_{max} (%)
IL-4	230 (140–390)	100 ± 7	5 (2.5–10)	100 ± 11	0.4 (0.1–1.6)	100 ± 6
Variant Y124F	150 (110–210)	97 ± 6				
Variant Y124H	190 (130–290)	82 ± 5				
Variant Y124N	220 (170–290)	29 ± 7				
Variant Y124K	230 (210–250)	20 ± 4				
Variant Y124G	130 (120–140)	13 ± 4	~40	~77	~5	~99
Variant Y124D		<0.5 ± 0.7	310 (160–620)	8 ± 3	~210 (120–360)	45 ± 6

EC_{50} values were distributed log normal. Numbers in brackets were calculated from log EC_{50} ± log SD.
R_{max} values ± SD were related to R_{max} of IL-4 as 100%.
Approximate values (~) were obtained from a single experiment.

T-cell proliferation assay (see Tables I and II). Thus, the properties of partial agonist Y124G are consistent with an IL-4 receptor system in B-cells operating at low occupancy during CD23 induction. In this case, the EC_{50} values for a partial agonist are expected to increase proportionally to the loss of efficacy (Ruffolo,Jr, 1982).

The CD23 positive cells represented 67% of the total cells analysed by FACS. This percentage remained constant at all saturation levels. Thus, the induction of the number of CD23 positive cells and the induction of mean CD23 content most probably reflected two aspects of the same process in the same cell population. Of two alternatives—either each induced cell immediately develops a full CD23 content or the whole population is first induced to develop a low CD23 content which upon higher stimulation is increased—the second is consistent with the observed data.

Competitive receptor binding

The dose–response curves suggest that the amino acid exchanges at position 124 did not dramatically alter the receptor affinity of the mutant proteins compared with wild type IL-4. This has been corroborated by radioligand receptor binding experiments (Figure 3). IL-4, as well as the mutant proteins studied here, competed efficiently with iodinated IL-4 for the IL-4 receptors present on the B-lymphoma cell line Raji (Cabrillat *et al.*, 1987; Park *et al.*, 1987) as shown in Figure 3 for Y124G and Y124D. The displacement curves of all variants, with the exception of Y124D, were not significantly different from that of IL-4 (see also Table II). Half-maximal displacement of iodinated IL-4 required a 3.4-fold higher concentration of mutant protein Y124D compared with that of IL-4. Corresponding competitive binding studies performed with both PHA-activated T-cells and activated tonsillar B-cells yielded similar relative IC_{50} values (Table II). (Unfortunately, the large numbers of activated B-cells necessary for radioligand binding could not routinely be obtained from human spleen).

The concentration of 1 nM [^{125}I]IL-4 during the competitive binding experiments was ~10-fold higher than the dissociation constant. Thus, the K_D of the competing ligands could not be determined from the IC_{50} (Munson, 1983). The K_D of [^{125}I]IL-4 determined independently (see Materials and methods) was 91 pM for the B-lymphoma cells and 81 pM for the PHA-activated T-cells. Assuming a receptor dissociation constant K_D of ~100 pM for IL-4 (see also Cabrillat *et al.*, 1987; Park *et al.*, 1987), mutant protein Y124D accordingly had a K_D of 310 pM.

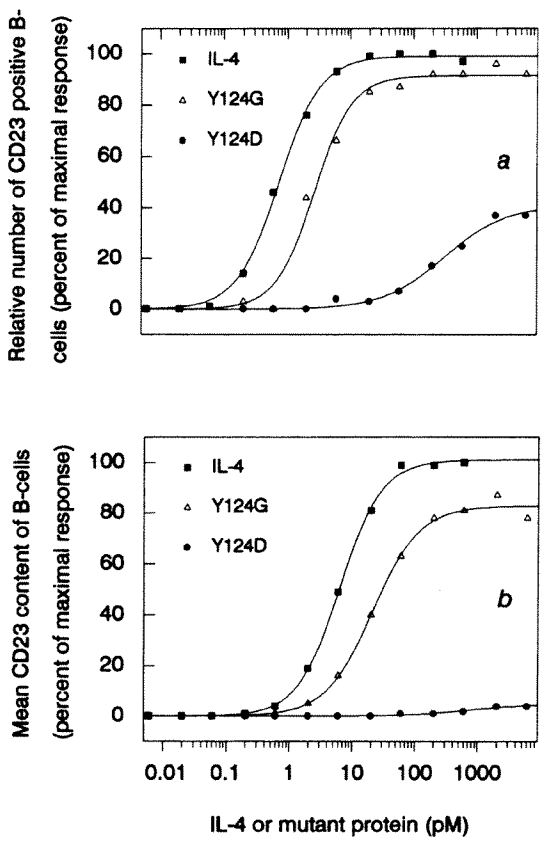

Fig. 2. Induction of the low affinity Fcε receptor (CD23) on B-cells in response to increasing concentrations of IL-4 (■) or mutant proteins Y124G (△) and Y124D (●). (**a**) Number of CD23 positive B-cells as percent of maximal response. (**b**) Mean CD23 content of B-cells.

Activated human B- and T-cells both expose 500–1000 molecules of IL-4 receptor at their surface showing a K_D of ~100 pM in terms of IL-4 binding (see above). Therefore the proliferative response of T-cells appears to closely follow the receptor occupancy. Assuming that in the B-cell assays the biological response is solely mediated by the receptor with a K_D of 100 pM, the induction of CD23 requires the occupancy of only a few percent of the receptors by IL-4. We suggest that only in case of the 'emasculated' (Black, 1989) Y124D mutant protein, the receptor had to be maximally saturated in order to generate a small partial response. It remains unclear, however, if the assay periods

Table II. Competitive radioligand binding (IC_{50}), receptor dissociation constant (K_D) and competitive inhibitor constant (K_i) of IL-4 and IL-4 variants evaluated from B- and T-cell assays

Protein	PHA-activated T-cells		B-cells			T-cell proliferation	B-cell (spleen)	
	IC_{50} (%)	K_D (pM)	Lymphoma (Raji)		Tonsillar	K_i(pM)	mean CD23 content	number of CD23 positive cells
			IC_{50} (%)	K_D (pM)	IC_{50} (%)		K_i (pM)	
IL-4	100 (80–130)	81 ± 7	100 (70–150)	91 ± 5	~100			
Variant Y124F			75 (70–80)					
Variant Y124H			80 (70–90)					
Variant Y124N			110 (100–120)					
Variant Y124K			110 (100–120)					
Variant Y124G			68 (60–80)					
Variant Y124D	320 (230–450)		340 (280–410)		~300	620 (390–990)	810 (620–1050) ~640[a]	1000 (320–3200) ~630[a]

IC_{50} and K_i values were distributed log normal. The IC_{50} of the variants were related to the IC_{50} of IL-4 as 100%. Numbers in brackets were calculated from log IC_{50} ± log SD or log K_i ± log SD.
K_D values ± SD were calculated from Scatchard analyses.
[a]Tonsillar B-cells were used in one experiment.

of several days conventionally used allow for a straight comparison between receptor occupancy and response.

Antagonist activity of IL-4 variant Y124D

The results presented above lead to the prediction that the mutant protein Y124D functions as a pure antagonist in the T-cell system and as a partial antagonist of IL-4 in the much more sensitive B-cell system. This has been actually demonstrated by competition experiments (Figure 4). IL-4-stimulated proliferation of T-cells was competitively inhibited by mutant protein Y124D (Figure 4A). From the apparent EC_{50} in the presence of different concentrations of Y124D an inhibitor constant K_i of 620 pM could be calculated (see Table II). A similar K_i of 810 pM was determined during competititon of IL-4-dependent CD23 induction on B-cells (Figure 4B). Included in Table II is also the inhibitory constant K_i of 1000 pM for the induction of the number of CD23 positive B-cells (Figure 4C). The latter value shows a high variability due to the pronounced partial agonist activity of variant Y124D during this response (45%). For the partial agonists Y124G, Y124N, Y124K and even for Y124H, and incomplete inhibition of IL-4 activity was established in the T-cell proliferation assay down to the level determined by their respective partial agonist activities (see Figure 1). The calculated K_i values were in the range of 200 pM (data not shown).

A comparison of the dose–response curves in Figure 4A, B and C shows that the slopes of the T-cell responses are steeper. The same difference was observed for the experimental results presented in Figures 1 and 2A, and B. The binding curve of [^{125}I]IL-4 to B-lymphoma cells or activated T-cells has a slope with a calculated Hill coefficient of 0.81–1.15 (data not shown) similar to the B-cell responses (0.91–1.4). The Hill coefficient calculated for the slope of the dose–response curve for IL-4-dependent T-cell proliferation is ~2-fold higher (2–2.7). In the competitive inhibition experiments, the slope was reduced ~2-fold in the presence of antagonist Y124D, both in the T-cell (1.3–1.5) and in the B-cell (0.5–0.6) assays. The significance of these differences is still unclear. They might also indicate specific aspects of receptor activation, but we cannot presently rule out an origin from experimental conditions.

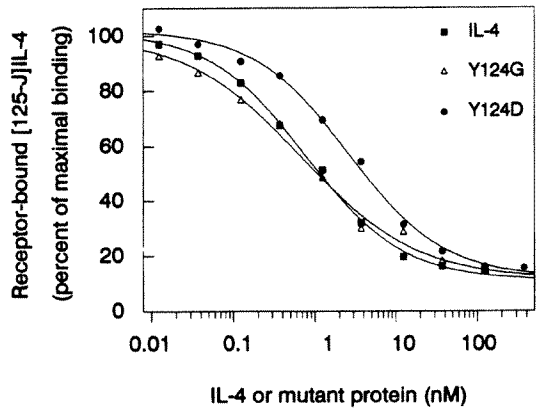

Fig. 3. Competition for binding to the IL-4 receptor of Raji cells between radiolabelled IL-4 and IL-4 (■) or mutant proteins Y124G (△) or Y124D (●).

Discussion

The results presented here indicate conclusively that Tyr124 of IL-4 is of crucial importance for the potency of the lymphokine for signal generation, but not for the binding of IL-4 to its receptor. Whereas biological activity was severely impaired in several substitution mutants at position 124 (see below), binding of this variant ligands to IL-4 receptor was not at all or only marginally affected. As compiled in Tables I and II, this is shown first by radioligand receptor binding experiments using the IL-4 receptor of the B-lymphoma cell line Raji (Park *et al.*, 1987), or of both activated T- and B-cells, secondly, by determining the EC_{50} of the partial agonist activities in both the B-cell and the T-cell assay and thirdly, by determining the competitive inhibitor constants, K_i, in both cellular systems.

The impairment of biological activity, i.e. the loss of efficacy of IL-4, strongly depends on the nature of the side chain introduced at position 124. In the T-cell assay, efficacy is reduced ~5-fold with side chains of asparagine, lysine and glycine, respectively, and >100-fold with an aspartyl side chain (see Table I). Interestingly, the loss of efficacy appears to be less pronounced during the B-cell differentiation assays. This, however, corresponds to the

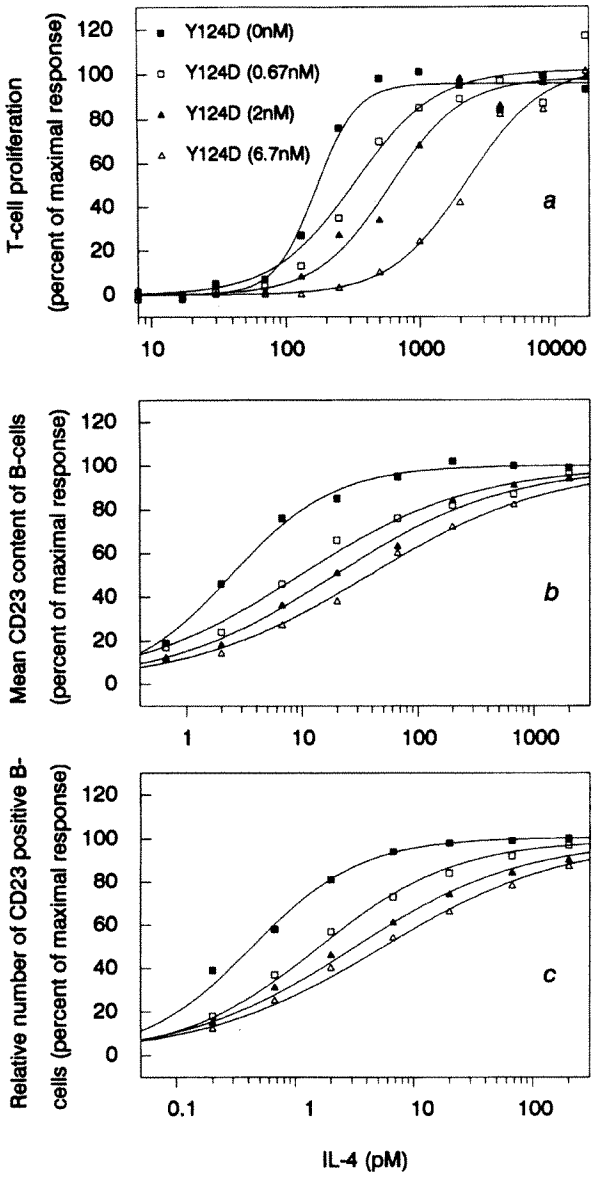

Fig. 4. Competitive inhibition of IL-4-dependent T-cell proliferation. (**a**) IL-4-induced CD23 content of B-cells (**b**) and IL-4-induced number of CD23 positive B-cells (**c**) by mutant protein Y124D at concentrations of 0 nm (■), 0.67 nM (□), 2 nM (▲), and 6.7 nM (△). The results in (B) and (C) are corrected by subtracting partial agonist activities of variant Y124D.

~40-fold lower EC_{50} of IL-4 for the mean CD23 induction or the 500-fold lower EC_{50} for induction of CD23 positive cells when compared with the T-cell response.

These apparent discrepancies in IL-4 efficacy can be explained in terms of receptor occupancy. The EC_{50} of ~200 pM for T-cell proliferation is similar to the IL-4 receptor dissociation constant. Accordingly, this response may be limited by the number of activated receptors. The B-cell response appears to be limited by a signalling step following receptor activation. This step then would determine the EC_{50} of 5 pM (0.4 pM) in these processes, which correspond to a receptor occupancy of ~5% (mean CD23 content) or 0.4% (number of CD23 positive cells). The calculation and its underlying assumptions are detailed in Materials and methods. The lower efficacy of the IL-4 variants can thus be partially compensated for by higher receptor saturation. In case of mutant protein Y124D maximal response is 8% (45%) with an EC_{50} or 310 pM (210 pM) for mean CD23 induction (CD23 positive cells). According to these data, the loss of efficacy is 125- to 300-fold. During the T-cell proliferation assays, the mean error of the control was 0.5% of the maximal response of IL-4. The responses of variant Y124D were within the mean error of the control. Thus, the efficacy of Y124D is at least 200-fold lower than that of IL-4 in this system. Although these calculations are rough estimates derived from long term assays, measuring complex proliferation and differentiation processes, at least they should provide information concerning the upper limit. (An alternative explanation of the results involving a very small subpopulation of receptors, e.g. 20–50 molecules/cell, with a very low dissociation constant in the range of 1 pM in the B-cells appears rather unlikely, since the antagonist Y124D inhibits with the same inhibitory constant of 620–1000 pM both B- and T-cells.)

The identification of a whole panel of partial agonists suggests that the residue at position 124 is directly involved in signal generating interactions. The amino acid exchange in mutant protein Y124D, however, produces an unexpectedly large effect. Thus, additional conformation effects appear to be possible in this case. It may be relevant in this respect that variant Y124D does not easily crystallize (T.Müller and W.Sebald, unpublished observation) in contrast to IL-4 (Cook *et al.*, 1991). It remains to be established if further residues in the IL-4 molecule, together with Tyr124, form a site distinct from a IL-4 receptor binding site, which is involved in signal generation. According to the available structural information (Redfield *et al.*, 1991; see also Diederichs *et al.*, 1991) residues at the C-terminal end of helix D are close to Tyr124. Cys127 forms a disulfide bond to Cys3. Thus, side chains of helix A may also be in proximity to Tyr124. It is interesting to note that for human GM-CSF, which has the same fold as IL-4, a receptor binding site was postulated consisting of two peptide stretches centered around Arg24 (helix A) and Met79 (helix C) (Diederichs *et al.*, 1991). According to the GM-CSF crystal structure, this binding site would be spatially far away from the C-terminus of helix D.

Tyr124 of human IL-4 is found at the corresponding position of murine IL-4 (Sanderson *et al.*, 1988). It occurs at the end of helix D (Redfield *et al.*, 1991), only six residues away from the C-terminus. It has been noted (Bazan, 1990b; Sanderson, 1988) that this C-terminal segment has features in common with other known ligands of the haematopoietin receptor family. Amino acid substitutions of Glu141 of murine IL-2 (Zurawski *et al.*, 1990), whose position appears to be equivalent to Tyr124 of human IL-4, yielded a series of partial agonists. In particular, murine IL-2 variant Q141D was inactive or only weakly active in proliferation assays using various cell lines. However, high affinity binding was lost and no competitive inhibition of IL-2-dependent proliferation of HT2 cells could be found even at a 5×10^4-fold molar excess of variant Q141D. Variant Q141D of murine IL-2 is a capable antagonist, however, against mutant proteins of IL-2 with receptor-binding defects. mIL-2 variants with substitutions of Glu141 by asparagine (class I), lysine (class II), aspartic acid (class III) and glycine

(class IV), could be grouped into four classes according to their differing partial agonist activities. This is unlike the variants of human IL-4, where substitutions of Tyr124 by asparagine, lysine and glycine produced very similar agonist activities (see Figure 1, Table I). Thus, it remains unclear in how far the generation of partial agonism by an aspartyl side chain at the human IL-4 Y124 and at the murine IL-2 Q141 positions indicates comparable structural requirements.

Two non-overlapping binding sites were identified in human growth hormone (Cunningham and Wells, 1991; Cunningham *et al.*, 1991; De Vos *et al.*, 1992) that are occupied in a sequential manner by two growth hormone receptor molecules. Site 1 comprises the C-terminus of helix D (Cunningham *et al.*, 1991). The equivalent of IL-4 Tyr124 might be human growth hormone Val185, which is located at the end of helix D and which is part of site 1. The biological activity of a growth hormone variant V185D has not yet been assessed. It was postulated (Cunningham *et al.*, 1991) that non-dimerizing variants of growth hormone, which have lost the ability to bind two receptor molecules, should show antagonistic properties.

A natural receptor antagonist was detected in the interleukin 1 system. The human protein (IL-1Ra) shares 19% identical amino acid positions with IL-1α and 26% identical positions with IL-1β and is probably composed of 12 β-strands. It behaves as a pure antagonist in binding with high affinity to the IL-1 receptor (type I) without evoking any detectable response (Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). This indicates that in the IL-1 system receptor binding and receptor activation are completely separable. An efficient uncoupling of both steps also was obtained by single amino acid replacements in IL-1 molecules. IL-1β variants R127G (Gehrke *et al.*, 1990) and D145K (Ju *et al.*, 1991) as well as an IL-1α variant D151Y (Yamayoshi *et al.*, 1990) exhibited little biological activity but bound with a largely unaltered affinity to the receptor. The discontinuous receptor binding site identified in human IL-1β (Labriola-Tompkins *et al.*, 1991) clusters around Arg4 and does not comprise the positions determining receptor activation. This could argue for a structural separation of two independent functional sites in the IL-1 protein. Interestingly, however, significant differences exist among those amino acid residues of IL-1α, IL-1β and Il-1Ra that probably provide the contact points in each receptor–ligand complex. Accordingly, an apparently unaltered receptor affinity of an engineered antagonist might also result from multiple compensating effects and it does not necessarily indicate a structural independence of the two ligand domains that bind or activate the receptor, respectively.

Especially for IL-1α variant D141G pure agonist or partial agonist activities were established in various cellular responses (Yamayoshi *et al.*, 1990), which resemble our results with human IL-4 variant Y124D. Considering the fundamental structural differences between IL-1 and IL-4, as well as between their respective receptors, it is interesting to speculate that the occurrence and the straight forward identification of high affinity antagonists in both systems is correlated with a single chain composition of both the receptors.

Other more complex receptor systems may put more stringent constraints on structural modifications of the ligand. In the multichain IL-2 receptor system the intrinsically high K_D of 70 nM of the β subunit (the homologue of the IL-4 receptor) was found to decrease to 5 pM in the presence of the α subunit (see Ringheim *et al.*, 1991). A decreased K_D of 1.2 nM was observed in YT cells due to the presence of a still poorly defined γ subunit. Interestingly, IL-2 variants affected in α subunit binding showed an apparently unaltered EC_{50} in the biological assays (Weigel *et al.*, 1989; Zurawski and Zurawski, 1989; Ju *et al.*, 1991). For the explanation of this puzzling behaviour a model was recently proposed (Grant *et al.*, 1992) according to which the α subunit contributes several functions to IL-2-mediated signalling through the high affinity IL-2 receptor system. Interestingly, the partial agonist and antagonist variants affected at position Gln141 of murine IL-2 (Zurawski *et al.*, 1990) were described to be deficient in γ subunit interactions. They inhibited efficiently, however, the IL-2 variants defective in α subunit binding. Possibly, the interaction of the γ subunit and the β subunit is critical for receptor activation and any disturbance of this interaction will reduce both receptor binding and receptor activation. This complex subunit interplay in the IL-2 receptor system therefore complicates its analysis, but may also provide special advantages for the engineering of selective agonists (see Zurawski, 1991).

Increasing evidence supports the view that the formation of receptor homodimers or the forming of heterooligomers represents an important step in receptor transmembrane signalling (see Schlessinger, 1988; Williams, 1989; Ullrich and Schlessinger, 1990). This leads to the question how the ligand triggers this aggregation. 'Allosteric receptor oligomerization' (Schlessinger, 1988), proposed as a model for tyrosine kinase receptors, involves two conformations of the extracellular domain. Only the high affinity ligand binding conformation forms oligomers. Bridging of two receptor proteins by a single ligand molecule represents another possible mechanism for oligomerization (Ullrich and Schlessinger, 1990; Cunningham *et al.*, 1991) supported by the recent finding that growth hormone forms a 1:2 complex with the isolated extracellular receptor domain. For the IL-4 receptor, no evidence for the occurrence of dimers or oligomers has as yet been provided. A single class of IL-4 receptor or IL-4 binding protein has been identified so far, exhibiting one homogeneous dissociation constant of ~100 pM. It remains to be established whether IL-4 receptor signalling involves the formation of a homodimer or whether the aggregation with other hitherto unknown proteins is necessary. The availability of antagonist IL-4 variants will open up new ways to address the question of which mechanisms operate during IL-4 receptor signalling.

Materials and methods

Production of IL-4 mutant proteins

Human IL-4 and mutant proteins were produced in *Escherichia coli*, subjected to a renaturation step and highly purified by CM-Sepharose 6B chromatography followed by HPLC (Weigel *et al.*, 1989; Kruse *et al.*, 1991). By means of *in vitro* mutagenesis Tyr124 (Kruse *et al.*, 1991) had been replaced by phenylalanine, histidine, asparagine, lysine, glycine or aspartic acid, respectively, to generate mutant proteins Y124F, Y124H, Y124N, Y124K, Y124G and Y124D. The mutated genes recloned into the expression plasmid were sequenced in both directions (373A DNA Sequencer, Applied Biosystems) to confirm the mutation. Protein concentration was determined by measuring absorbance at 280 nm. It was assumed that 1 mg IL-4/ml (50% Acetonitril and 0.1% trifluoracetic acid) yields an absorbance of 1 at 280 nm ($\epsilon_{280}^{0.1\%} = 1$). For protein measurements of variants deficient in Try124, the absorbance at 280 nm was multiplied by a factor of 1.15.

T-cell proliferation assay

(see Yokota *et al.*, 1986; Solari *et al.*, 1989) Peripheral blood mononuclear cells obtained from healthy donors were purified by Ficoll–Hypaque centrifugation (Pharmacia) and stored in aliquots at −80°C. The thawed cells were cultured for 7 days with 9 μg/ml phytohemagglutinine (PHA; HA-15, Wellcome) and consisted at this stage to a high percentage of activated T-cells (PHA-blasts). Cells (5×10^5/ml) were incubated in 0.2 ml aliquots with log 2 dilutions of the IL-4 protein for 3 days, before the amount of [^{3}H]thymidine incorporated during the final 4 h was determined. Maximal response was $6-23 \times 10^3$ c.p.m. incorporated [^{3}H]thymidine over a background of 55–1400 c.p.m. The EC_{50} values were calculated from the known IL-4 concentration of a stock solution and the dilution factor yielding half-maximal response.

Induction of the low affinity Fcε receptor (CD23) on B-cells

(see Kikutani *et al.*, 1986; DeFrance *et al.*, 1987; Solari *et al.*, 1989) Mononuclear cells were purified from the spleen of a donor that had no haematological disease by centrifugation over Ficoll–Hypaque. B-cells were isolated by rosetting of contaminating cells with ox erythrocytes coupled with monoclonal antibodies anti-CD2, anti-CD4 and anti-CD8 . Purified B-cells were positive by >95% for CD19. Tonsillar B-cells were prepared in the same way. B-cells were cultured at 10^6 cells/ml for 40 h with 10 μg/ml soluble F(ab')$_2$ fragments of affinity purified goat anti-human IgM (Jackson Laboratories-Dianova, Hamburg, Germany) plus log 3 dilutions of IL-4 or mutant proteins. FACS analysis (FACScan, Becton-Dickinson) was done after double-staining with monoclonal antibodies against CD19 (Leu12-PE, Becton-Dickinson) and CD23 (10B8-FITC, Dianova). The number of CD23 positive cells and the relative mean fluorescence intensity was calculated from a histogram obtained from the single cell analysis of 5000 cells evaluated over a 4 log range. B-cells without stimulation were CD23 positive by <1% with a relative mean fluorescence intensity of <10. After maximal stimulation 67 (62–72)% of the cells were CD23 positive exhibiting a relative mean fluorescence intensity of 795 ± 11. EC_{50} values were calculated from the known concentrations of the IL-4 stock solution and the dilution factor leading to half-maximal response.

Receptor binding assays

[^{125}I]IL-4 labelled to a specific radioactivity of 0.22 μCi/pmol (Cabrillat *et al.*, 1987) was incubated at a concentration of 1 nM with 10^7/ml Raji B-lymphoma cells or activated T-cells or activated tonsillar B-cells, respectively, in 0.2 ml plus the indicated concentrations of the competing IL-4 proteins. The [^{125}I]IL-4 radioactivity bound during a 2 h incubation period at 4°C was determined. Dissociation constants, K_D, were calculated from relative IC_{50} according to Ruffolo (1982). Measurements of [^{125}I]IL-4 binding to receptors on PHA-activated T-cells and activated tonsillar B-cells was performed as described above but using varying concentrations (10–1000 pM) of [^{125}I]IL-4 in the absence and presence of 10 μM unlabelled IL-4, respectively.

Competitive inhibition assays

Activated T cells (PHA-blasts) were incubated with log 2 dilutions of IL-4 (see above) plus a constant amount of mutant protein Y124D. B-cells (CD19 positive cells) were incubated with log 3 dilutions of IL-4 (see above) at a constant concentration of mutant protein Y124D. The CD23 content of the cells was analysed by FACS and evaluated as described above. The K_i of variant Y124D was calculated from the apparent EC_{50} values measured in the presence of two different inhibitor concentrations [I^2] and [I^1] according to the relation:

$$K_i = \frac{[I^2] - EC_{50}{}^2/EC_{50}{}^1 \times [I^1]}{EC_{50}{}^2/EC_{50}{}^1 - 1} \quad \text{(Ruffolo, 1982)}$$

Calculations of receptor occupancy and relative efficacy

The receptor occupancy y is the ratio between the concentrations of liganded receptor [RL] and total receptor [R_t]. Making the most simple assumption that one ligand binds to one receptor with one homogeneous dissociation constant K_D then

$$y\% = \frac{[RL] \times 100}{[R_t]} = \frac{[L] \times 100}{K_D + [L]}$$

It is further assumed that the dissociation constant K_D of 10^{-10} determines the IL-4-dependent CD23 induction in B-cells. Then the receptor occupancy y for IL-4 is 5% (0.4%) at the EC_{50} of 5 pM (0.4 pM) for the induction of mean CD23 content (number of CD23 positive cells).

The partial agonist Y124D probably saturates the receptor (y = 100%) in B-cells to produce the maximal partial response, i.e. the induction of 8% (45%) of the full mean CD23 content (number of CD23 positive cells).

The relative efficacy (e_r) of two ligands is the ratio of the responses R^1 and R^2 that would be produced by these ligands at the same receptor occupancy.

$$e_r = \frac{R^1}{R^2} \times \frac{y^2}{y^1}$$

Accordingly, for IL-4 and variant Y124D the relative efficacy amounts to 50/8 × 100/5 = 125 (50/45 × 100/0.4 = 275) during induction of mean CD23 content (number of CD23 positive cells).

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THE CD4 RECEPTOR: POST BINDING EVENTS, CONFORMATIONAL CHANGE AND THE SECOND SITE

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The human differentiation antigen CD4 is expressed on a variety of cell types, in particular T lymphocytes, dendritic cells and cells of the macrophage/monocyte lineage. Its expression on T lymphocytes identifies a subset of cells which are predominantly associated with helper/inducer functions, which are depleted in AIDS (Schroff *et al.*, 1983) and which show class II restriction; that is, they respond to antigen only when it is presented by cells bearing homologous MHC class II molecules.

The CD4 antigen is also established as the major cellular receptor for the human immunodeficiency virus (HIV) (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984). Viral tropism is mediated by the high affinity interaction between CD4 and the major viral envelope glycoprotein gp120. Human CD4- cells which are normally resistant to infection can be rendered susceptible if they are induced to express CD4 by transfection with the gene encoding CD4. Fusion is mediated by the N-terminal domain of the smaller viral envelope glycoprotein, gp41. However, CD4 expression alone is not sufficient to allow HIV infection to occur, since mouse cells transfected with human CD4 are not susceptible (Maddon *et al.*, 1986).

This observation led to the concept of a "second site" - that is, that property of human CD4+ cells which allows them to be infected once virus binding has occurred, and which is not present in murine CD4+ transfectants. Various suggestions have been made regarding the possible nature of the second site:

(i) another cell surface protein, for example MHC class I or II, to which the virus envelope or CD4 would have to bind before fusion with the cell membrane could occur.

(ii) a site on CD4, distinct from the site mediating the high affinity interaction with gp120 which has been localised to the N-terminal domain (see below), which would interact in some way with the virus to facilitate fusion, and which is available on native CD4 but not on the molecule as expressed by the murine transfectant.

(iii) a non-protein characteristic of the human cell membrane, such as lipid composition, which renders possible interaction with the fusogenic region of gp41.

(iv) a conformational change in CD4, only possible in the context of the native molecule and not

possible when it is expressed in the murine transfectant cell membrane.
Several recent studies support the suggestion that the CD4 molecule itself is involved in post-binding events leading to fusion of the virus with the cell membrane.

Firstly, there is good evidence that CD4 has a signal transduction function, and does not act simply as an adhesion molecule increasing the affinity of the MHC class II/T cell receptor interaction, raising the possibility that CD4 undergoes conformational changes during this interaction. Secondly, two recent studies have suggested that gp120 binding and cell-cell fusion are mediated by different regions of the CD4 molecule (Camerini and Seed, 1990; Hillman *et al.*, 1990). Anti-CD4 monoclonal antibodies (MAbs) have been described which block syncytium formation and virus infection without blocking the binding of gp120 (Healy *et al.*, 1990). Thirdly, it has been shown that there is no correlation between the ability of anti-CD4 MAbs to inhibit syncytia and their ability to block gp120/CD4 binding (Wilks *et al.*, 1990). Fourthly, it has been found that some anti-CD4 MAbs enhance the binding of others to CD4 in a way which is best explained by the induction of a conformational change in CD4 after binding (Walker *et al.*, 1991). Finally, X-ray crystallographic studies suggest that the area of CD4 involved in binding to gp120 is a flexible exposed loop in the N-terminal domain of the molecule (Wang *et al.*, 1990; Ryu *et al.*, 1990).

This chapter examines the possible nature of the second site in the light of this recent experimental evidence regarding the structure and function of CD4. These findings, which will be discussed in detail below, support the suggestion that CD4 is involved in post-binding events that lead to fusion, and that a conformational change in the molecule may be part of this function.

(1) The CD4 Molecule: Structure and Function

CD4 is a non-polymorphic 55 kD glycoprotein consisting of four tandem extracellular domains which contain significant sequence and structural homology with the variable and joining regions of immunoglobulin supergene family members. There is a short hydrophobic transmembrane region and a cytoplasmic tail (Maddon *et al.*, 1985).

Analysis of mature T cells indicates that they segregate broadly into two classes: those that express CD4 and respond to antigen only in the context of MHC class II - so called "MHC class II restriction" - and those that express CD8 and show MHC class I restriction. It is now firmly established that CD4 binds to non-polymorphic parts of MHC class II molecules (Sleckman *et al.*, 1987; Gay *et al.*, 1987; Doyle and Strominger, 1987); it is thought that CD4 associates with the T-cell receptor/CD3 complex (TCR/CD3) conferring class II restriction by increasing the avidity of the T cell/antigen-presenting cell interaction (reviewed in Germain, 1988; Janeway, 1989).

There is also evidence that CD4 may have other functions, such as signal transduction. Anti-CD4 MAbs may in some circumstances suppress T cell activation in systems where accessory cells do not carry MHC class II (Banks and Chess, 1985). Carrel *et al.*, (1988) showed that CD4+ cells can be activated by the anti-CD4 MAb B66, and that conversely this MAb can inhibit the specific cytolytic activity of CD4+ effector cells. CD4 is associated with the internal membrane tyrosine-kinase $p56^{lck}$ (Veilette *et al.*, 1988, 1989). Cross linking of CD4 is associated with the rapid phosphorylation of the ζ-subunit of the TCR/CD3 complex and a rapid increase in tyrosine-specific protein kinase activity. Thus, in addition to interacting directly with class II molecules, these data provide evidence for a specific $p56^{lck}$-mediated CD4 signal transduction pathway.

(2) The Role of CD4 as the Receptor for HIV

Selective depletion of peripheral CD4+ lymphocytes, and an inversion of the normal CD4/CD8 ratio

were noted to be features of AIDS shortly after the syndrome was first described (Gottlieb *et al.*, 1981; Schroff *et al.*, 1983). Therefore, when HIV was isolated, it was suspected that the virus might display selective tropism for CD4+ cells. This was shown to be the case by Klatzmann *et al.*, (1984). HIV induces non-viable multi-nucleate giant cells (referred to as "syncytia") in lymphocyte cultures *in vitro*. Dalgleish *et al.*, (1984) showed that of 135 MAbs to human leukocyte cell surface proteins tested, only MAbs to CD4 could inhibit the formation of syncytia and infection of cells by vesicular stomatitis virus (VSV) pseudotypes bearing HIV surface antigens. Anti-CD4 MAbs also prevented the productive infection of T cells as assessed by RT production (Klatzmann *et al.*, 1984). It was therefore concluded that the CD4 molecule forms at least part of the HIV receptor.

McDougal *et al.*, (1986a) demonstrated by immunopreciptation that HIV binds to CD4+ cells by binding of the viral envelope protein gp120 to CD4. It was also shown that after binding of gp120 the epitope on CD4 defined by the MAb OKT4A was no longer available whereas the epitope for OKT4 remained available. The same group (McDougal *et al.*, 1986b) demonstrated direct binding of HIV particles to CD4+, but not CD4- cells. The involvement of the gp120/CD4 interaction in the mechanism of syncytium formation was shown by several groups who demonstrated that syncytia forming in mixed cultures do not involve CD4- cells (Lifson *et al.*, 1986a), that syncytia can form between CD4+ cells and CD4- cells that have been transfected with the HIV env gene alone (Sodrowski *et al.*, 1986), and that syncytia can form in CD4+ cells infected with a recombinant vaccinia virus containing the HIV envelope gene alone (Lifson *et al.*, 1986b).

Following the isolation of the gene encoding CD4, it was possible to transfect CD4- cells that had previously been uninfectable, and it was found that in human cells, but not in mouse cells, expression of CD4 was sufficient to confer infectability (Maddon *et al.*, 1986). Conversly, modulation of CD4 by phorbol ester leads to loss of susceptibility to infection (Clapham *et al.*, 1987). Fusion of the virus with the host cell membrane has been shown to occur by a pH independent mechanism (Stein *et al.*, 1987; McClure *et al.*, 1988). The cytoplasmic domain of CD4 is not required for infection, since CD4- human T cell lines transfected with mutant CD4s lacking the cytoplasmic tail became infectable after transfection (Maddon *et al.*, 1988; Bedinger *et al.*, 1988). A CD4/CD8 chimaera containing only the amino-terminal 177 residues of CD4 functioned as an effective virus receptor in the latter system.

Epitopes of CD4

Considerable effort has since been directed towards determining which epitopes on CD4 are involved in gp120 binding in order to further elucidate the mechanism of HIV infection. This has involved antibody studies and experiments involving mutant forms of CD4. Both lines of enquiry have yielded evidence suggesting that CD4 is involved in fusion, not just as a passive receptor, and that critical residues for this lie outside the area generally believed to be involved in direct contact with gp120.

(i) Inhibition of Virus Binding/Infection by Anti-CD4 MAbs

McDougal *et al.* (1986b) showed that OKT4A, OKT4D, OKT4F and anti-Leu3a but not OKT4 blocked binding of HIV to CD4+ cells. In order to map those epitopes of CD4 required for viral attachment, Sattentau *et al.* (1986) examined a large panel of MAbs for their ability to inhibit syncytia. However, in many cases, MAbs were only available as ascites, and therefore a formal comparison of efficacy on a molar basis was not made. In spite of this constraint, the results obtained were broadly consistent with those of McDougal *et al.* The ability of excess unlabelled MAb to cross compete for CD4+ cells with small quantities of a limited range of high affinity radioiodinated MAbs was also used, to group MAbs into clusters that mutually inhibit one another. It was found that antibodies that inhibit syncytia fall into at least two groups which do not cross compete, and therefore presumably recognise

separate epitopes. Thus anti-Leu3a and OKT4A mutually inhibit, as do MT151 and Vit4, but the first two do not inhibit the last two, whilst all four inhibit syncytia.

Lundin *et al.* (1987) examined the ability of MAbs at known concentration to inhibit the binding of purified native gp120 to CD4+ cells. This study showed a similar result to the two previous reports, with the important exception that OKT4C was able to partially block binding at moderately high concentrations. Similar results were obtained by Lamarre *et al*. (1989), who examined the ability of MAbs to block binding of recombinant gp120 to CD4-transfected cells, using an indirect flow cytometry method. It was shown that OKT4C, D, E and F could all be blocked by large excesses of gp120.

It is likely that blocking assays and the syncytium inhibition assay are both dependent to some extent on antibody affinity. It is important therefore that these assays are performed with known concentrations of MAb, and that the interpretation of results takes differences in relative affinity into account. Thus, Lamarre *et al.* (1989) found it necessary to use much more gp120 to block binding of OKT4E and OKT4F than was needed for OKT4C. For these reasons, the studies of McDougal *et al.* (1986b) and Sattentau *et al.* (1986) do not really contribute to identification of the gp120 binding site, apart from establishing that differences exist between MAbs.

Antibodies that Block Syncytia but not gp120 binding

Healy *et al.* (1990) have described two novel anti-CD4 MAbs designated Q425 and Q428 which prevent HIV infection of cells and inhibit syncytium formation at low concentration without blocking gp120 binding. In fact they reproducibly induce a small enhancement in gp120 binding. Furthermore, critical residues for these antibodies have been mapped to amino-acids 178-292, an area corresponding to the third extracellular domain of CD4 (V_3) distant from the gp120 binding site in V_1. Preincubation of CD4 with gp120 reduces the affinity of these antibodies. The authors speculate that these MAbs bind to V_3 and stabilise the CD4 molecule thus preventing a subsequent conformational change necessary for fusion and syncytium formation. Preincubation with gp120 may induce a conformational change in V_3, thus accounting for the reduction in MAb binding. Whilst this interpretation remains speculative, it supports the hypothesis that gp120 binding alone is not sufficient for CD4 to take part in fusion.

(ii) Epitope Mapping Studies based on Mutant CD4s

Epitope mapping studies based solely on blocking by anti-CD4 MAbs can demonstrate that MAbs are different (although it appears that the majority can inhibit gp120 binding at least partially), but they do not yield evidence about the topographical location of epitopes on the CD4 molecule. More sophisticated epitope mapping studies have therefore used mutant forms of CD4, incorporating truncations and amino acid deletions, insertions and substitutions. These studies have often combined mapping of the gp120 binding site with mapping of anti-CD4 MAb epitopes, thus allowing information from the former studies to be used more effectively. Two assumptions underlie the use of mutant CD4s; firstly, that the effect of a mutation in the primary sequence produces only local changes in tertiary structure. This can sometimes be confirmed by demonstrating broad preservation of MAb epitopes. Secondly, it is often assumed that if mutation of a given residue abrogates binding of a ligand, that residue is a contact residue, or lies close to contact residues, for binding of that ligand. This is not necessarily so, and it is more appropriate to refer to such residues as "critical residues".

Truncation Mutants

Reliable but limited evidence of epitope location may be obtained by studying the activity of truncated forms of CD4. Berger *et al.* (1988) produced a soluble recombinant fragment of CD4 consisting of the amino-terminal 180 residues, thus containing only the V_1 and V_2 domains. This fragment reacted with all anti-CD4 MAbs tested except OKT4. This fragment also bound gp120, and this was taken as evidence that the gp120 binding site lies within the V_1 and V_2 domains. A similar result was obtained by Bedinger *et al.* (1988), who made chimaeric membrane-located mutants between CD4 and CD8, and showed that the N-terminal 177 residues of CD4 can bind HIV efficiently.

Capon *et al.* (1989) and Traunecker *et al.* (1989) have synthesised "immunoadhesins" - soluble recombinant molecules that bear only the two N-terminal domains of CD4, spliced to constant regions of human immunoglobulins (IgG and IgM respectively). These constructs efficiently neutralise all strains of HIV yet tested.

Landau *et al.* (1988) constructed chimaeric forms of CD4, in which part or parts of the molecule were substituted with analogous parts of the murine homologue. A construct consisting of murine amino acids 1-37, followed by the normal human sequence, was able to bind gp120. Similarly a construct consisting of murine amino acids from position 159 onwards bound CD4. It is likely that the effect of substitutions on such a large scale is to produce widespread changes in conformation. For this reason, as with most studies involving the use of mutants, conservation of function is a much more reliable observation than loss of function. Chao *et al.* (1989) subsequently produced a recombinant fraction of CD4 consisting only of the V_1 domain, residues 1-113, and this was shown to bind gp120 as efficiently as full length SCD4.

Localised Mutations

In order to locate epitopes more precisely within the V_1 domain various groups have constructed CD4 mutants with more localised changes in primary sequence. Petersen and Seed (1988) generated mutant CD4 cDNAs incorporating point mutations and short deletions throughout the V_1 domain. Mutations affecting gp120 binding were located at residues 45, 46 and 47. gp120 binding was also abrogated by a 42-49 deletion mutant. The same mutations also resulted in loss of syncytium formation; reduced syncytium formation was noted with mutations at residue 40 and a double substitution at residues 40 and 45. Much information was also obtained about the binding sites of anti-CD4 MAbs. These patterns, together with previous evidence from MAb competition studies, were used by the authors to confirm the validity of modelling CD4 onto an immunoglobulin variable region domain. The putative gp120 binding site maps to the area corresponding to the C' and C'' strands of the Ig variable region, or the second hypervariable region (CDR2).

A similar result was obtained by Clayton *et al.* (1988), who constructed mutant forms of CD4 incorporating substitutions from the murine homologue. Mizukami *et al.* (1988) used site-directed mutagenesis to construct mutant CD4s with two amino acids inserted at various points throughout the V_1 and V_2 domains. Hydrophilic amino acids were inserted in hydrophilic, and therefore presumably surface exposed areas, and residues were carefully chosen for insertion in order to minimise disruption of secondary structure. Insertions affecting gp120 binding were located at 31, 44, 48, 55 and 57.

Arthos *et al.* (1989) constructed a panel of soluble recombinant mutants incorporating mouse CD4/ human CD4 substitutions and examined their ability to bind gp120 with a variety of assays, most of which were at least semi-quantitative. Mutations affecting gp120 binding were again located to the region analogous to the second hypervariable or complementarity determining region (CDR2), at residues 40-43, 50-53 and 55. The same panel of mutants was used by Sattentau *et al.* (1989) to

map the epitopes of a large panel of anti-CD4 MAbs. Results were broadly consistent with previous work.

Ashkenazi *et al.* (1990) produced a series of soluble mutants of CD4 based on alanine-scanning mutagenesis, which is one of the most complete studies to date. Several mutations outside the area homologous to CDR2 (in particular residues 29, 77-81 and 85) markedly affected gp120 binding without affecting the binding of a panel of anti-CD4 MAbs, suggesting that overall conformation was not disrupted.

Brodsky *et al.* (1990) examined a panel of cell surface expressed mutants of CD4. Mutations outside the CDR2 homologous area did not affect gp120 binding except in one case in which binding of all V_1 reactive MAbs was also lost. However, mutations in several parts of the CDR2 homologous area (residues 58, 59 and 62) disrupted gp120 binding, suggesting that an extensive part of that area is involved in binding to gp120.

The studies reviewed above are in general agreement that the gp120 binding site on CD4 involves the mid-portion of the V_1 region, homologous to CDR2. Although specific criticisms can be brought against each of these methods, in many instances there is general agreement on the location of MAb epitopes on CD4. However, the instances in which they differ tend to involve MAbs whose affinity is comparatively low such as OKT4E (Wilks *et al.*, 1990). A knowledge of relative functional antibody affinity is therefore important in the correct interpretation of such studies since it allows explanation of apparent anomalies and may also suggest limitations to the value of the information obtained.

Two studies, however, suggest that areas of the V_1 region outside the gp120 binding site are involved in fusion.

Camerini and Seed (1990) have shown that substitution of residue 87, an amino acid which is not considered to be part of the gp120 binding site, with the analogous residue from the chimpanzee CD4, destroys the ability of cells to form syncytia without affecting binding of gp120. Certain anti-gp120 MAbs also prevent syncytium formation but not gp120 binding (Linsley *et al.*, 1988).

Hillman *et al.* (1990) have described the derivation of a series of mutants from the human cell line CEM using a chemical mutagen followed by negative selection with anti-CD4 MAbs. Various mutant cell lines were derived among which were CD4+ cells which were able to bind gp120 but were unable to form syncytia after infection with HIV or a gp120-vaccinia recombinant. The nature of the mutation in CD4 that gave rise to this phenomenon was not elucidated.

This apparent separation of the ability to bind gp120 from the ability to form syncytia based on changes in the structure of CD4 is in accordance with the findings, reviewed above, that some anti-CD4 MAbs are able to inhibit syncytium formation without blocking gp120 binding.

(3) Epitope Mapping and Affinity

Wilks *et al.* (1990) examined the relationship between the functional affinity of a panel of anti-CD4 MAbs and their ability to inhibit HIV-induced syncytia and block the binding of gp120 to CD4+ cells, since assays based on these properties have formed the basis of the epitope mapping studies reviewed above.

The measurement of affinity of MAbs for large cell surface molecules presents practical difficulties. Classical methods of affinity measurement, such as equilibrium dialysis, are ruled out by the size

of the antigen (Mason and Williams, 1986). An inhibition radioimmunoassay (RIA) was therefore used, based on the observation that in solid phase assays, the concentration of soluble ligand required to prevent binding of antibody to immobilised ligand is inversely related to antibody affinity (Andersson, 1970; Rath *et al.*, 1988).

There was a strong correlation between the functional affinity of anti-CD4 MAbs and their ability to inhibit syncytia, and a weak correlation between functional affinity and ability to block gp120 binding, but no correlation between the ability to inhibit syncytia and the ability to block gp120 binding. The majority of MAbs that blocked syncytia well also completely blocked gp120 binding, but MAbs that inhibited syncytia only at high concentrations only partially reduced gp120 binding. This effect could be due to steric hindrance but it is also possible that CD4 undergoes an allosteric change post binding of the anti-CD4 MAb, which reduces the affinity of the gp120-CD4 interaction.

There are several interesting anomalies in this data. For example, antibody L218 is a high affinity antibody that inhibits syncytia at 4μg/ml. However, it only blocks binding of gp120 to 50%, even at 200 μg/ml. In contrast, OKT4D is a moderate affinity MAb that inhibits binding of gp120 100% at 0.8μg/ml, but only inhibits syncytia at 240 μg/ml. L216 is able to block syncytia completely at 220 μg/ml, but it achieves only 34% reduction in gp120 binding at that concentration.

These discrepancies between gp120 blocking and syncytium inhibition suggest that cell fusion and gp120 binding are functions of distinct parts of the CD4 molecule.

(4) Competition and Enhancement between Anti-CD4 MAbs

Competition assays, in which different MAbs are allowed to interact simultaneously with a single ligand, have been used for epitope mapping in many fields. It is normally assumed that the ability to inhibit binding indicates identity of binding sites. When this technique was used with solid phase SCD4 and a panel of anti-CD4 MAbs, it was found that binding of some, but not all, high affinity MAbs to solid phase immobilised SCD4 greatly enhanced the binding of several lower affinity MAbs. A more limited pattern of enhancement was seen when CD4+ cells were used as the antigen (Walker *et al.*, 1991).

Enhancement, which is a specific quality of particular pairs of antibodies, could occur through a variety of mechanisms, most of which remain speculative:

(i) Enhancement could be a non-specific physical effect due, for example, to viscosity or protein concentration. This is unlikely because of the specific nature of the pairing; MAbs enhance some MAbs and inhibit others.

(ii) Enhancement could be mediated by cross-linking of antigen by the first MAb facilitating bivalent binding by the labelled MAb. Cross-linking is not a likely mechanism for this effect, since it is also mediated by Fab fragments, and the antigen density used was sufficiently low to make bivalent binding unlikely.

(iii) Enhancement might be due to specific binding of one MAb by another - anti-idiotypic binding between anti-CD4 MAbs would not be inconceivable. However, no such binding could be demonstrated.

(iv) This effect could be the result of conformational change in the CD4 molecule induced by the

binding of one MAb. Levels of antibody binding in solid phase assays are known to be influenced by antibody affinity (Lew, 1984). It is possible that binding of one MAb to CD4 induces a conformational change that increases the affinity of the labelled MAb. This might be due to stabilisation, or decreased flexibility of CD4, favouring one of many possible available conformations, or it could be due to the induction of a novel conformation. Healy *et al.* (1990) report a similar enhancement of gp120 binding by the MAbs Q425 and Q428 (see above).

Whilst the mechanism of enhancement remains unclear, as a phenomenon it is helpful for grouping MAbs for epitope mapping, since it is unlikely that MAbs that enhance each other share binding sites, and it is likely that MAbs that show similar patterns of inhibition and enhancement bind to CD4 in similar ways. Similar experiments have been used in the past to define groups of MAbs that mutually cross inhibit and the assumption has been made that these groups of MAbs share common or overlapping epitopes (Sattentau *et al.*, 1986, 1989; Jameson *et al.*, 1988). Since some MAbs can enhance the binding of others, it follows that the same effect might mediate *reduction* in binding of one MAb by another and lead to an erroneous assumption that the two MAbs share epitopes.

(5) Atomic Structure of CD4

X-ray crystallographic structures of V_1V_2 fragments of CD4 have recently been published (Wang *et al.*, 1990; Ryu *et al.*, 1990). As was suggested by sequence analysis, both N-terminal domains closely resemble the immunoglobulin light chain variable region in their folding patterns. Each domain is an anti-parallel β-barrel with features characteristic of an immunoglobulin fold. The two domains pack against each other with a large common hydrophobic interface. The area of V_1 associated with gp120 binding ("the C" ridge") forms an exposed loop, suggesting that the complementary site on gp120 is likely to be a groove-like structure.

These crystal structures explain many of the apparent minor anomalies in the epitope mapping studies reviewed above. For example residues 20 and 90 are shown to lie in close proximity, accounting for the fact that mutations at these apparently separate sites affect binding of the same MAb. Interpreted in conjunction with mutational analyses, the crystal structures suggest that MAbs MT151 and Vit4 bind to a face formed by the V_1V_2 domains on the opposite side to the C" ridge, whereas OKT4A, OKT4D and anti-Leu3a bind around the ridge in different positions. The high thermal parameters of the C" ridge suggest that it is likely to be a flexible structure. The identification of this region is an important advance both for understanding of the gp120/CD4 interaction and for the design of inhibitors.

Conclusion

In summary, evidence has been presented that gp120 binding and syncytium formation are separate functions that both depend on the CD4 molecule. MAb studies and mutational analyses identify different parts of the molecule as being required for these two processes. Evidence has also been presented that the CD4 molecule is a mobile molecule that can undergo conformational change during normal physiological function, after gp120 binding and after MAb binding. X-ray crystallography has identified the gp120 binding site as a flexible exposed ridge.

It seems likely that CD4 not only functions as a passive receptor for HIV, but also plays a part in post binding events leading to fusion. The extent to which this involves conformational change remains to be established.

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An Antibody to Lymphotoxin and Tumor Necrosis Factor Prevents Transfer of Experimental Allergic Encephalomyelitis

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Summary

Uncertainty regarding pathogenic mechanisms has been a major impediment to effective prevention and treatment for human neurologic diseases such as multiple sclerosis, tropical spastic paraparesis, and AIDS demyelinating disease. Here, we implicate lymphotoxin (LT) (tumor necrosis factor β [TNF-β]) and TNF-α in experimental allergic encephalomyelitis (EAE), a murine model of an autoimmune demyelinating disease. In this communication, we report that treatment of recipient mice with an antibody that neutralizes LT and TNF-α prevents transfer of clone-mediated EAE. LNC-8, a myelin basic protein–specific T cell line, produces high levels of LT and TNF-α after activation by concanavalin A, antibody to the CD-3ϵ component of the T cell receptor, or myelin basic protein presented in the context of syngeneic spleen cells. LNC-8 cells transfer clinical signs of EAE. When LNC-8 recipient mice were also treated with TN3.19.12, a monoclonal antibody that neutralizes LT and TNF-α, the severity of the transferred EAE was reduced, while control antibodies did not alter the disease. The effect of anti-LT/TNF-α treatment was long lived and has been sustained for 5 mo. These findings suggest that LT and TNF-α and the T cells that produce them play an important role in EAE.

It has been suggested that the cytokines lymphotoxin (LT)[1] (TNF-β) and TNF-α could contribute to pathogenesis in several human neurologic diseases, including multiple sclerosis (MS) (1) and AIDS dementia (2). If that were the case, inhibition of the induction or activity of such cytokines might alleviate the tissue damage and demyelination associated with these diseases. In this study, we test the hypothesis that LT and TNF-α are involved in experimental allergic encephalomyelitis (EAE), a murine model for MS, and demonstrate that inhibition of their activity prevents transfer of clinical signs of this paralytic disease.

LT and TNF-α are genetically related cytokines with several activities that could contribute to demyelinating diseases. TNF-α is produced by macrophages after stimulation with LPS, whereas both LT and TNF-α are released by T cells activated by antigen or infection with some viruses, including human T cell leukemia virus type I (HTLV-I) (3, 4). LT and TNF-α activities appear to be beneficial in defense against tumors and virus-infected cells (3), and detrimental in their association with cachexia (5) and (for TNF-α) in the pathogenesis of cerebral malaria (6). Evidence has accumulated that supports but does not yet prove a role for LT and TNF-α in certain neurologic diseases. One of the earliest descriptions of LT was derived from a study of lymphocytes of rats with EAE (7). TNF-α causes demyelination and death of oligodendrocytes in vitro (8). Further corroboration and a suggestion for the biologic relevance of these observations is indicated by the recent description of TNF-α in MS plaques (9). The high levels of LT produced by HTLV-I-infected T cell lines (10–12) is also consistent with an involvement of LT in tropical spastic paraparesis, a neurologic disease associated with infection with that human retrovirus.

In related studies (13, 14), we have presented circumstantial evidence for a role of LT in EAE. A series of myelin basic protein (MBP-reactive) PL/J T cell clones had identical antigen fine specificity and MHC restriction, used the same TCR Vβ gene (15), and produced IL-2 in response to the encephalitogenic peptide (amino acids 1–11) of MBP presented in the context of H-2^u. Despite these similarities, the clones varied in their ability to transfer EAE, and this was positively correlated with the amount of LT/TNF-α cytotoxic activity and

[1] *Abbreviations used in this paper:* EAE, experimental allergic encephalomyelitis; HTLV-I, human T cell leukemia virus type I; LT, lymphotoxin; MBP, myelin basic protein; MS, multiple sclerosis.

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the amount of LT mRNA that they produced. Clones that transferred EAE efficiently, as assessed by incidence of disease, severity of clinical signs, and day of onset, produced high levels of cytotoxic factors (256 U) and mRNA for LT. Those that did not transfer disease produced low or undetectable levels (0–16 U) of cytotoxic activity and LT mRNA. The correlation with TNF-α was not as clear. Most clones that made LT mRNA also made TNF-α mRNA. However, one clone made high levels of TNF-α mRNA, but did not secrete cytotoxic material, and did not transfer disease particularly well. No correlation was found between IFN-γ and IL-2 production and encephalitogenicity.

In this communication, we present additional data that support the hypothesis that LT and/or TNF-α are involved in the pathogenesis of EAE. These results obtained with the SJL/J murine model of EAE indicate that inhibition of LT and TNF-α reduces the severity of disease symptoms transferred by a T cell line. One implication of these results is that inhibition of these cytokines might alleviate clinical signs in certain immunologically mediated human neurologic diseases.

Materials and Methods

LNC 8 T Cell Line. LNC-8 was derived from the popliteal lymph nodes of an SJL mouse immunized with porcine MBP. LNC-8 was maintained with biweekly addition of human rIL-2 (Amgen Biologicals, Thousand Oaks, CA) and stimulated with porcine MBP (10 μg/ml; Calbiochem-Behring Corp., San Diego, CA) and irradiated SJL splenocytes every other week.

Activation of LT and TNF-α Production. LNC-8 cells (2×10^6/ml) were incubated in medium that contained 10% FCS alone, or were supplemented with Con A (5 μg/ml), or 25 or 100 μg/ml porcine MBP and irradiated syngeneic SJL spleen cells. For stimulation with anti-CD3, LNC-8 cells were added as above to tissue culture flasks that had been incubated overnight with anti-CD3 ϵ antibody YCD3-1 (16), then rinsed twice with PBS. Culture supernatants were collected from activated cells at various times between 8 and 48 h and tested for cytotoxic activity against WEHI 164 cells.

Biologic Assay for LT and TNF-α. For determination of LT/TNF units, a sensitive WEHI 164 subline obtained from Edward Lattime (Memorial Sloan-Kettering, New York) was used as a target. WEHI 164 cells (5×10^3/well) were set up in 96-well tissue culture plates in RPMI 1640 with 10% FCS, 1% nonessential amino acids, 1% essential amino acids, 1% sodium pyruvate in dilutions of test and control samples in a volume of 100 μl. After 44 h, WEHI 164 survival was evaluated by the cells' 4-h uptake of MTT (17), which was then acidified with 150 μl 0.04M HCL in isopropenol. The plate was read on a Vmax plate reader at 470 nm with a 650-nm reference standard. Units were calculated as the highest dilution causing 50% cytotoxicity. Percent cytotoxicity was calculated as: 100× (1.00 − OD of sample wells)/OD of control wells.

Neutralization of LT/TNF-α Biologic Activity. Supernatants containing WEHI 164 cytotoxic activity were diluted to 64 U and incubated with TN3.19.12 obtained from Dr. Robert Schreiber (Washington University, St. Louis, MO). This hamster mAb has been previously demonstrated to neutralize both LT and TNF-α (18). After incubation for 1 h at 37°C, the supernatants were added to WEHI 164 cells and assayed as above.

Northern Blot Analysis. Total RNA was obtained by the guanidinium thiocyanate cesium chloride method (19) from unstimulated murine L cells, and from SJL splenocytes and LNC-8 cells after activation. RNA (20 μg) was subjected to electrophoresis in a 0.66 M formaldehyde/MOPS 1% agarose gel, transferred to Gene Screen Plus (New England Nuclear, Boston, MA), and hybridized with TNF-α cDNA (20) (a generous gift from Dr. Bruce Beutler, University of Texas Southwestern Medical School), LT cDNA (21), or β-actin cDNA (22) (a generous gift from Dr. D. Cleveland, Johns Hopkins University Medical School) labeled by the random primer method (23). The filters were washed twice for 15 min at 55°C with 0.2× SSC and 0.1% SDS, and exposed to XAR film with intensifying screens at −70°C.

Transfer and Evaluation of EAE. LNC-8 cells were stimulated with MBP (10 μg/ml) and spleen cells for 3 d. The cells were then exposed to 2 U/ml IL-2 for 1 d. After that time, $7.5–12 \times 10^6$ LNC-8 cells were injected intraperitoneally or intravenously as indicated in individual experiments. The mice were divided into groups and, 48 h later, injected with PBS, hamster control antibody L2D39, rat anti-IL-4 antibody 11B11 (24), or hamster anti-LT/TNF-α antibody TN3.19.12 (18). Mice were evaluated for clinical signs of EAE daily for at least 21 d after injection of cells. The study was done in a blinded fashion. That is, the observer was unaware of the protocol. The clinical scale was as follows: 0 = normal, 1 = tail limpness, 2 = paraparesis with a clumsy gait, 3 = hind limb paralysis, 4 = hind and fore limb paralysis, 5 = death.

FACS Analysis. LNC-8 cells were analyzed for Vβ gene usage 12 d after being stimulated with irradiated splenocytes and MBP. Cells were incubated with supernatant from KJ23a, a generous gift of Drs. John Kappler and Phillippa Marrack (National Jewish Center, Denver, CO), or a control supernatant and stained with fluorescein-conjugated goat anti-mouse antibody. Binding was evaluated with a FACS analyzer (Becton Dickinson & Co., Mountain View, CA).

Results

Characteristics of LNC-8 Cells. The IL-2-dependent T cell line LNC-8 used in the present studies to transfer EAE proliferates in response to MBP in the context of $H\text{-}2^s$. It proliferates to all three major pepsin-digested fractions of MBP (data not shown). One of these fractions includes the peptide, which is encephalitogenic for SJL/J mice, that lies in the COOH-terminal end of the MBP molecule (25). We have found by FACS analysis that ~50% of LNC-8 T cells stain with KJ23a, an antibody that reacts with the Vβ17a gene product of the TCR (Fig. 1). This TCR gene usage is consistent with the observation that Vβ17a and at least one other as yet undefined Vβ gene are utilized by encephalitogenic SJL clones (26).

LT and TNF-α Production by LNC-8 Cells. When LNC-8 cells are stimulated by Con A, they produce mRNA for several cytokines, including IFN-γ and GM-CSF (data not shown) and LT and TNF-α (Fig. 2). Of particular interest is the fact that in the RNA samples from stimulated LNC-8 cells LT mRNA appeared to be at higher abundance than TNF-α mRNA, and that the T cell line made considerably more LT and TNF mRNA than do Con A–activated spleen cells. There are actually several molecular species of LNC-8 RNA that hybridize with both the LT and TNF-α cDNA probes. One high molecular weight species (~3 kb) hybridizes with both probes and could represent an LT promoter-regulated transcript driven through the entire 6-kb

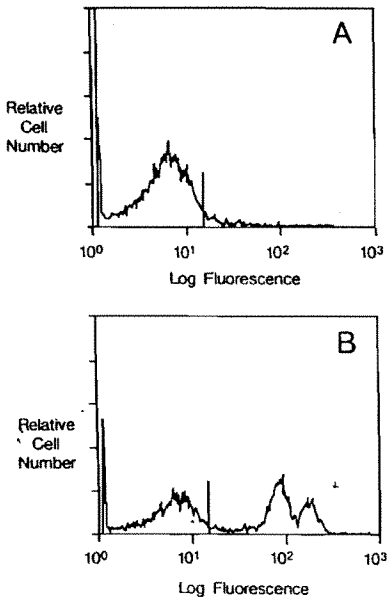

Figure 1. The LNC-8 cell line includes a Vβ17a$^+$ population. LNC-8 cells were analyzed for Vβ17a usage 12 d after last being stimulated with irradiated SJL splenocytes and MBP. Cells were incubated with supernatant KJ23a (anti-Vβ17a) or with control supernatant, washed, and stained with fluorescein-conjugated goat anti-mouse antibody. Binding was analyzed on a FACS analyzer (Becton Dickinson & Co.). (*A*) Control supernatant; (*B*) anti-Vβ17a.

TNF-α/β gene complex within the MHC on mouse chromosome 17 (27, 28).

Culture supernatants from LNC-8 cells activated by several different methods contained cytotoxic activity against WEHI 164 cells, a target for both LT and TNF-α (Table 1). Because the bioassay does not distinguish between the two cytokines, the results are expressed as LT/TNF-α activity. LNC-8 cells did not secrete LT/TNF unless activated. They secreted high levels of LT/TNF after stimulation with nonspecific mitogens, stimulation through the TCR by an anti-CD3 antibody YCD3-1 (16), or by MBP presented by syngeneic spleen cells. The amount of cytotoxic activity in the culture supernatants increased over time of stimulation, particularly when LNC-8 cells were activated with MBP and spleen cells. In experiment 1 (Table 1), the amount of cytotoxic activity in supernatants after antigen stimulation was 256 U at 8 h, 1,024 U at 24 h, and 2,048 U at 48 h. Northern blots of RNA prepared from LNC 8 cells activated under all conditions of Table 1 were positive for both LT and TNF-α mRNA, with usually higher accumulations of LT mRNA, as in Fig. 2. Though it is not possible to distinguish between LT and TNF-α in the biologic assay, it is likely that the WEHI 164 killing in the experiments reported here is due to both cytokines since the activated LNC-8 cells made both mRNAs. In other studies, we have observed that the kinetics of LT and TNF-α mRNA production can vary in individual T cell clones, and the cytotoxic activity attributable to the individual cytokines varies with activation time.

Table 1. *LNC-8 Cells Produce LT/TNF After Activation*

Exp.	Stimulating agent*	LT/TNF‡
1	–	0
	MBP/SC	256
	Con A	32,000
2	–	2
	MBP/SC	512
	Anti-CD3	25,600

* LNC-8 cells were incubatd for 8 h in growth media plus 10% FCS. Conditions of stimulation were as indicated in Materials and Methods. Exp. 1, MBP concentration was 25 μg/ml; Exp. 2, MBP was 100 μg/ml.
‡ LT/TNF units were determined by cytotoxic effect against WEHI 164 cells.

Neutralization of LNC-8 Cytotoxic Activity by Anti-LT/TNF-α Antibody. In a previous publication (18), we described a hamster mAb, TN3.19.12, that reacts both with LT and TNF-α derived from supernatants of T cell clones and with TNF-α derived from macrophage culture supernatants. This antibody also neutralized the cytotoxic activity secreted by LNC-8 cells, indicating that the WEHI 164 killing activity is due to LT and/or TNF-α. TN3.19.12 anti-LT/TNF-α antibody completely neutralized WEHI 164 cytotoxic activity of supernatants obtained after all methods of activation of LNC-8 cells, including antigen plus spleen cells (Fig. 3), Con A, and anti-CD3 antibody.

Inhibition of Transfer of EAE by LNC-8 by Treatment of Recipient Mice with Anti-LT/TNF-α Antibody. LNC-8 cells are potent mediators of EAE. When 1.5 $\times$ 10^7 cells are injected

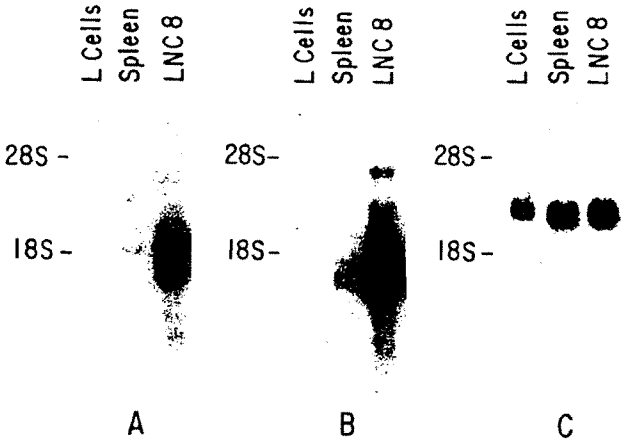

Figure 2. LNC-8 cells produce TNF-α and LT (TNF-β) mRNA. Northern blot analysis of total RNA (20 μg) obtained from unstimulated murine L cells and from SJL splenocytes and LNC-8 cells after 8-h exposure to 5 μg/ml Con A, hybridized with TNF-α cDNA (*A*), LT cDNA (*B*), or β-actin cDNA (*C*), and exposed with intensifying screens for 15 h (*A*), 2.5 h (*B*), or 30 min (*C*) to XAR-5 film.

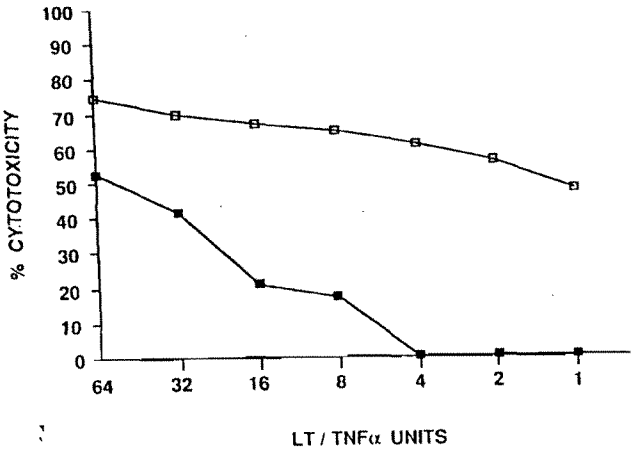

Figure 3. Hamster anti-LT/TNF-α mAb TN3.19.12 neutralizes cytotoxic activity in supernatants obtained from LNC-8 cells. Supernatant obtained from LNC-8 cells after incubation for 24 h with MBP and irradiated SJL spleen cells contained 1,024 U LT/TNF-α. The supernatant was diluted to 64 U and incubated in complete medium (□) or 850 ng TN3.19.12 (■) for 1 h at 37°C, and then added to WEHI 164 cells. Cytotoxicity was evaluated at 44 h.

intraperitoneally or intravenously, ~90% of the recipients develop clinical signs of severe EAE, usually within 7 d. Approximately 75% of the mice die. When lower numbers of LNC-8 cells are injected, the disease is not as debilitating, resulting in somewhat less severe clinical signs, fewer deaths, and delayed onset.

When mice that received LNC-8 cells were treated with the anti-LT/TNF-α mAb TN3.19.12, symptoms and severity of EAE were alleviated. In five different experiments (Table 2, protocol A), groups of four or five mice were injected with 7.5–9 × 10^6 LNC-8 cells intraperitoneally, and then treated 48 h later with a single intraperitoneal injection of 300 μg TN3.19.12, PBS, or a control hamster mAb, L2D39. Mice were evaluated daily for clinical signs of EAE for 3 wk. Treatment with anti-LT/TNF-α resulted in a dramatic reduction in the severity of clinical symptoms (Table 2). The effect of anti-LT/TNF-α treatment on the course of the disease is most apparent when the average disease scores of TN3.19.12-treated and control groups are compared. The average highest group score of mice that received 7.5–9 × 10^6 LNC-8 cells and PBS or the control hamster monoclonal L2D39 was 2.6 or 2.2, respectively, while the average highest group score of mice that received TN3.19.12 was 0.9, which is actually below clinically detectable disease (Table 2). These results are highly significant ($p < 0.001$) when TN3.19.12-treated mice were compared with PBS-treated mice by student's t test. L2D39 control (hamster antibody) treatment did not significantly affect the progression of the disease ($p < 0.252$). Those mice that did develop disease in the group receiving cells and TN3.19.12 developed clinical signs slightly later than did the control mice, and this disease was less severe. Those TN3.19.12-treated mice that did develop clinical signs had an average maximum disease score of 2, compared with 3.7 for mice that were treated with control antibody and developed clinical signs. In an additional experiment (Table 2, protocol B), 1.2 × 10^7 cells were injected intravenously in groups of 9 or 10 mice. In this experiment, a higher dose (1 mg) of anti-LT/TNF-α and a different control antibody (11B11, anti-IL-4) were used. The more severe disease that was induced with

Table 2. *Treatment of Mice with Anti-LT/TNF-α Antibody Reduces EAE Severity*

Protocol	Antibody treatment				Day of onset*	Average disease‡
	Amount	Antibody	n	Antibody specificity		
	mg					
A (7–9 × 10^6 LNC-8 cells i.p.)	–	PBS	10	–	6.2 ± 0.3	2.6 ± 0.4
	0.3	L2D39	15	–	7.8 ± 1.0	2.2 ± 0.7
	0.3	TN3.19.12	21	Anti-LT/TNF-α	8.6 ± 0.7	0.9 ± 0.3
B (1.2 × 10^7 LNC-8 cells i.v.)	–	PBS	9	–	7.1 ± 0.9	3.9 ± 0.6
	1	11B11	4	Anti-IL-4	7.5 ± 0.5	3.3 ± 1.6
	1	TN3.19.12	10	Anti-LT/TNF-α	7.0 ± 0.0	0.2 ± 0.1

$p < 0.001$ when 0.3 mg TN3.19.12 mice are compared with PBS-injected mice. $p < 0.2521$ when L2D39 mice are compared with PBS-injected mice. $p < 0.00035$ when 1 mg TN3.19.12-treated mice are compared with PBS mice. Protocol A was a summary of five separate experiments with four to five mice per group. Protocol B was one experiment.

* Average day of onset ± SEM of clinical signs of EAE.

‡ Average disease per group ± SEM. Mice were graded on a scale of 0–5, as indicated in Materials and Methods.

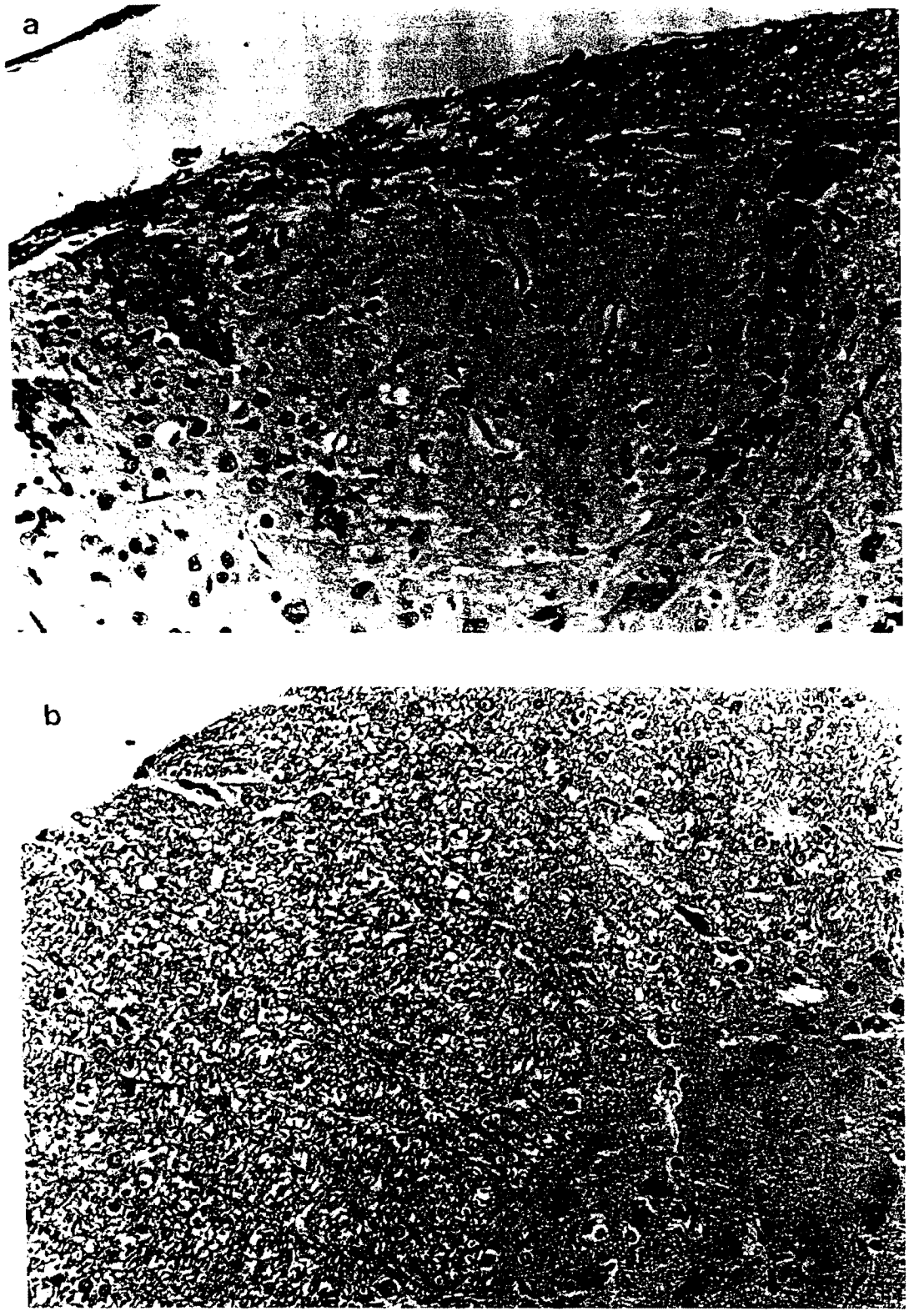

Figure 4. Anti-LT/TNF-α antibody treatment alleviates histologic signs of EAE. Analysis of spinal cord stained with hematoxylin and eosin. (*a*) Tissue section 9 d after injection of 9 × 10^6 LNC-8 cells and 7 d after PBS. Note perivascular infiltrate. (*b*) Tissue section 9 d after injection of 9 × 10^6 LNC-8 cells and 7 d after injection of 300 μg TN3.19.12. Note absence of infiltrate.

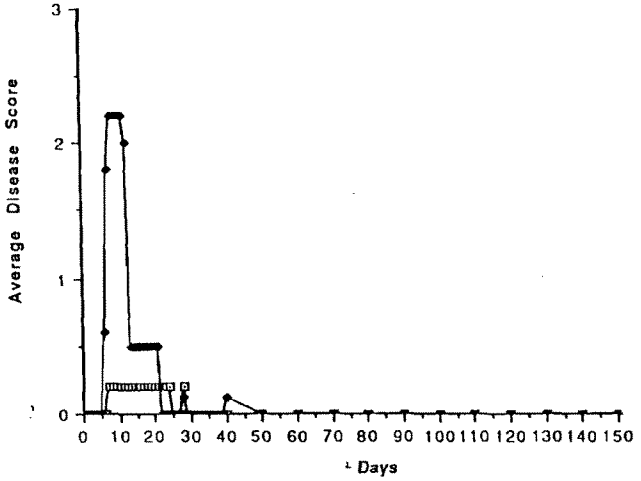

Figure 5. The alleviation of clinical signs of EAE induced by treatment with anti-LT/TNF-α antibody is persistent. Mice were injected with 7.5 × 10^6 LNC-8 cells intraperitoneally. 2 d later, one group of mice received PBS (◆), and one group received 300 μg anti-LT/TNF-α antibody TN3.19.12 (⊡). Mice were evaluated for clinical signs of EAE daily for 3 wk, and then three times a week for an additional 4 mo.

this dose of cells was dramatically inhibited with one injection of anti-LT/TNF-α. As indicated in Table 2, the average disease score was 3.9 for the PBS-treated group; 3.3 for the anti-IL-4-treated group, and 0.2 for the anti-LT-TNF-α treated group (the latter score again below clinically detectable disease). The incidence of disease was also reduced from 89% in the PBS treated group to 20% in the anti-LT/TNF-α group. The highest score of disease was 1 in this group.

Histological evaluation of mice that received LNC-8 cells and TN3.19.12 that remained clinically normal revealed that some of these mice showed no evidence of cellular infiltration or abnormality, while others showed meningeal infiltration and mild perivascular cuffing. All mice from the group that received LNC-8 cells and control antibodies or PBS that were examined had histologic signs of cuffing and infiltration consistent with their clinical signs (Fig. 4).

The inhibition of transfer of EAE by treatment with anti-LT/TNF-α antibody was long lived. Several groups of mice were observed for 2–3 mo. None developed disease if it had not occurred by day 12. In the representative experiment depicted in Fig. 5, mice were observed for 5 mo after receipt of 7.5 × 10^6 LNC-8 cells and antibody or PBS. The only mouse in the group of five that received both cells and TN3.19.12 to develop clinical signs had a limp tail (grade 1) from day 7 through day 26 and then recovered. Neither it nor any of the other mice in the antibody-treated group developed any further symptoms. In the control group (five mice) that received cells and PBS, four developed clinical signs, one of these died on day 12, and the others eventually recovered.

Discussion

The data presented here implicate LT and TNF-α in the passively transferred autoimmune neurological disease EAE. We demonstrate that a MBP-specific T cell line produces LT and TNF-α cytotoxic activity after activation with any of several different agents. The biologic activity is neutralized by an anti-LT/TNF-α antibody. The ability of LNC-8 cells to transfer EAE into naive mice is prevented if those mice are treated with the anti-LT/TNF-α antibody, but not with control antibodies. The use of an antibody that completely neutralizes both LT and TNF-α may be important since both cytokines are made by LNC-8 cells after activation with MBP, the relevant neuroantigen in this model of EAE. Absolute certainty that only one or the other cytokine is involved will only come with mAbs that distinguish between the murine forms. These are not yet available, and the polyclonal antibodies that we have tested in vitro are variable and inconsistent. It is particularly relevant to the human situation that we were able to inhibit the transfer of EAE by a cell line that contains at least two T cell populations that utilize different TCR-β genes. This suggests that these experiments may be broadly relevant to the human situation where, even though particular TCR-β genes may be preferentially utilized by siblings with MS (29), the actual gene(s) have not yet been identified. It is likely that the number of TCR-β genes utilized in this disease by the highly polymorphic human population is limited, but it is certainly greater than one.

The studies reported here are the first antibody neutralization experiments of EAE that provide insight into the identity of the mediators of the disease. The extent of prevention of transfer of EAE was dependent on the dose of anti-LT/TNF-α antibody used, and these doses (0.3 or 1 mg) were comparable with or less than those used in experiments with other antibodies to inhibit transfer or development of EAE. Sriram et al. (30) used three injections of 3 mg of anti-I-A antibodies on successive days before and after injection of encephalitogenic T cell clones to inhibit transfer of EAE. Urban et al. (31) injected 500 μg anti-Vβ8 antibody to prevent development of EAE, and Acha Orbea et al. (15) used two 100-μg injections of anti-Vβ8 antibody to inhibit T cell transfer of disease. In the previous experiments, the design was based on preventing T cell activation (30) or a major component of the T cell repertoire (15, 31). The experiments reported here represent a different approach to EAE; that is, TN3.19.12 inhibits the activity of mediators that are produced in vivo after stimulation of an antigen-specific population of effector T cells.

The mechanism by which the anti-LT/TNF-α antibody prevents the transfer of EAE is under investigation. It is possible that the antibody inhibits the disease in part by binding to and eliminating TNF-bearing T cells. This possibility is raised by the identification of membrane-bound TNF on a murine CTL clone with a polyclonal rabbit anti-TNF antibody (32), and the demonstration of TNF/cachectin on activated normal human T cells with an anti-TNF mAb (33). We do not believe that this is the most likely explanation for our results, because we have not been able to detect membrane TNF from macrophage lysates with TN3.19.12 (18), though we were able to detect a high molecular weight species from membranes of PU.5.1.8 with a polyclonal antibody (T. James and N. Ruddle, unpublished results). Moreover,

we have not been able to demonstrate surface staining by FACS with TN3.19.12 of LNC-8 cells treated identically to those used in the transfer studies. Maximum staining of 10^6 cells treated with 1, 10, or 100 μg/ml TN3.19.12 and a rabbit anti–hamster fluoresceinated antibody was 3.1%; under the same conditions, 94% of the cells were positive by anti-CD3. Because the concentration of TN3.19.12 antibody used in FACS experiments was comparable with that used in the in vivo experiments (i.e., 300 μg/7 $\times$ 10^6 cells), we believe it is unlikely that elimination of TNF-bearing cells is the mechanism. Nevertheless, this possibility exists and is under investigation in in vivo experiments with labeled cells. Histological analysis of tissue from mice that have received LNC-8 cells and TN3.19.12 has revealed that some treated, clinically normal mice had minimal evidence of cellular infiltrates, while others did not. Thus, the antibody may inhibit transfer at various stages by inhibiting several different activities of the LT and TNF-α secreted by LNC-8 cells. These activities involve a number of effects relevant to the pathogenesis of EAE, including an increase in MHC determinants on endothelial cells (34), an alteration of central nervous system permeability, and an influx of inflammatory cells through an increase in cell adhesion molecules on the endothelium (34) or astrocytes (35). Cytokines produced by these inflammatory cells may also directly affect the myelin sheath and influence viability of oligodendrocytes (8). TN3.19.12 antibody could inhibit any of these activities by directly neutralizing secreted LT and TNF-α. Further insight into these questions will derive from experiments underway designed to reverse established disease. Whatever the mechanism(s) by which this antibody inhibits transfer of EAE, this study demonstrates that inhibition of LT and TNF-α biologic activity prevents transfer of the symptoms of a severe an often fatal neurologic disease. These studies suggest a role for these cytokines or cells that express them in some neurologic diseases. If the transfer of disease in the EAE model is inhibited through inhibiting LT or TNF-α activity, the studies point the way for the development of TNF antagonists in such disease. The recent cloning of a receptor for LT and TNF-α (36, 37) elicits cautious optimism regarding feasibility of therapy for cytokine-mediated neurologic disease.

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Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin

(septic shock/cachectin/lymphotoxin/immunoglobulin chimera)

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ABSTRACT **Tumor necrosis factors (TNF) α and β are structurally related cytokines that mediate a wide range of immunological, inflammatory, and cytotoxic effects. During bacterial infection of the bloodstream (sepsis), TNF-α induction by bacterial endotoxin is thought to be a major factor contributing to the cardiovascular collapse and critical organ failure that can develop. Despite antibiotic therapy, these consequences of sepsis continue to have a high mortality rate in humans. Here we describe a potent TNF antagonist, a TNF receptor (TNFR) immunoadhesin, constructed by gene fusion of the extracellular portion of human type 1 TNFR with the constant domains of human IgG heavy chain (TNFR-IgG). When expressed in transfected human cells, TNFR-IgG is secreted as a disulfide-bonded homodimer. Purified TNFR-IgG binds to both TNF-α and TNF-β and exhibits 6- to 8-fold higher affinity for TNF-α than cell surface or soluble TNF receptors. *In vitro*, TNFR-IgG blocks completely the cytolytic effect of TNF-α or TNF-β on actinomycin D-treated cells and is markedly more efficient than soluble TNFR (24-fold) or monoclonal anti-TNF-α antibodies (4-fold) in inhibiting TNF-α. *In vitro*, TNFR-IgG prevents endotoxin-induced lethality in mice when given 0.5 hr prior to endotoxin and provides significant protection when given up to 1 hr after endotoxin challenge. These results confirm the importance of TNF-α in the pathogenesis of septic shock and suggest a clinical potential for TNFR-IgG as a preventive and therapeutic treatment in sepsis.**

Tumor necrosis factors α (TNF-α; cachectin) and β (TNF-β; lymphotoxin) are related proteins, secreted by activated macrophages and lymphocytes, respectively (1–3). These cytokines have been implicated in diverse biological processes including immunoregulation, inflammation, antiviral defense, cachexia, angiogenesis, and septic shock. The biological effects of TNF-α and TNF-β are mediated through specific receptors. Molecular cloning has demonstrated the existence of two distinct types of TNF receptor (TNFR), each of which binds to both TNF-α and TNF-β (4–8). The extracellular portions of both receptors are found naturally also as soluble TNF binding proteins (7, 8).

Several lines of evidence indicate that TNF-α is a principal mediator in the pathogenesis of septic shock. First, neutralizing anti-TNF-α antibodies can prevent the pulmonary failure and death associated with administration of endotoxin or *Escherichia coli* in mice (9) or baboons (10). Second, intravenous infusion of TNF-α leads to a toxic syndrome indistinguishable from that caused by endotoxemia and gram-negative sepsis (11, 12). In addition, the levels of TNF-α increase substantially in the circulation of animals and humans who have received endotoxin or have septic shock (13, 14) and correlate with mortality in severe sepsis (15–17).

To create a TNF antagonist that might block the lethal effect of TNF in endotoxic shock, we constructed an immunoadhesin (18) containing the extracellular portion of human type 1 TNFR and the hinge and Fc regions of human IgG heavy chain (TNFR-IgG). This approach was based on the observation that the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), can be linked to IgG heavy chain, thus creating a protein with two functional HIV binding sites and a markedly longer plasma half life than the soluble extracellular portion of CD4 (18, 19). We show that the TNFR-IgG chimera acts as a potent antagonist of TNF-α and TNF-β *in vitro* and can prevent endotoxin-induced lethality in a mouse model for septic shock. These results suggest that TNFR-IgG may be useful against the potentially lethal consequences of sepsis in humans.

MATERIALS AND METHODS

Construction, Expression, and Purification of TNFR-IgG. The schematic structure of TNFR-IgG is shown in Fig. 1*A*. A mammalian expression vector encoding TNFR-IgG (pRK-TNFR1-IgG) was constructed from plasmids encoding the human type 1 TNFR (pRK-TNF-R) (4) and CD4-IgG (pRKCD4$_2$Fc$_1$) (19). A 770-base-pair (bp) DNA fragment containing 5′ untranslated sequences and encoding the leader and extracellular portion of type 1 TNFR was generated by digesting pRK-TNF-R with *Eco*RI and *Hin*dIII. Plasmid pRKCD4$_2$Fc$_1$, encoding the extracellular domain of CD4 fused to the hinge and Fc region of human IgG$_1$ heavy chain (19), was digested with *Eco*RI and *Nde* I to remove most of the CD4 sequence while retaining the IgG$_1$ sequence. The TNFR-encoding fragment was then inserted 5′ of the IgG$_1$ sequence and in the same reading orientation by ligating the respective *Eco*RI sites and by blunting and ligating the *Hin*dIII and *Nde* I sites. The remaining CD4 sequence was removed to create the exact junction between threonine-171 of TNFR and aspartic acid-216 of IgG$_1$ heavy chain by oligonucleotide-directed deletional mutagenesis, using synthetic oligonucleotides complementary to the 24 nucleotides at the borders of the desired TNFR, and IgG$_1$ fusion sites as primers and the plasmid described above as a template (18). The final DNA construct was sequenced to confirm the correct primary structure. The mature TNFR-IgG polypeptide encoded by pRKTNFR-IgG thus contains 171 residues from TNFR and 227 residues from IgG$_1$—i.e., a total of 398

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Abbreviations: TNF, tumor necrosis factor; TNFR, TNF receptor; C_H, heavy-chain constant region; V_H, heavy-chain variable region; TNFR-IgG, fusion of TNFR with IgG$_1$ heavy-chain hinge region and C_H2 and C_H3 domains; HIV, human immunodeficiency virus; sTNFR, soluble TNFR.

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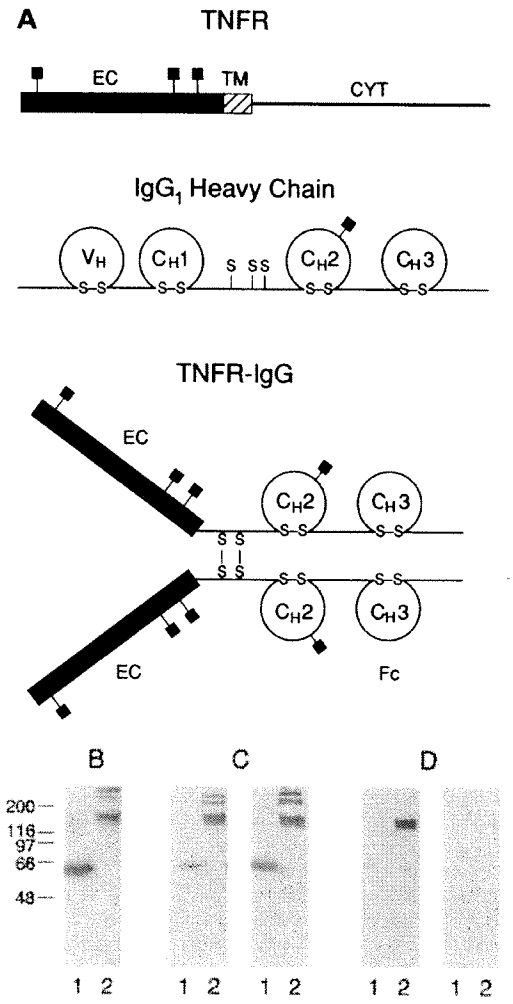

FIG. 1. (*A*) Schematic structure of the human type 1 TNFR, human IgG_1 heavy chain, and TNFR-IgG. The extracellular (EC), transmembrane (TM), and cytoplasmic (CYT) domains of TNFR and the IgG_1 heavy-chain variable region (V_H) and constant region (domains C_H1, C_H2, and C_H3) are indicated. TNFR-IgG was constructed by gene fusion of the extracellular domain of TNFR with the hinge region and C_H2 and C_H3 domains of the IgG_1 heavy chain. Potential asparagine-linked glycosylation sites (■) and disulfide bonds (S-S) (IgG protein only) are shown also. (*B–D*) Subunit structure and functional domains of TNFR-IgG. Human embryonic kidney 293 cells were transfected with a vector directing transient expression of TNFR-IgG. The protein was recovered from culture supernatants and purified by affinity chromatography on *S. aureus* protein A. SDS/polyacrylamide gel electrophoresis was carried out under reducing (lane 1) or nonreducing (lane 2) conditions. The proteins were stained with Coomassie blue (B) or electroblotted onto nitrocellulose paper and incubated with antibodies to human TNFR (*C Left*) or human IgG Fc (*C Right*) or with ^{125}I-TNF-α (1 nM) in the absence (*D Left*) or presence (*D Right*) of unlabeled TNF-α (100 nM). Blots were developed with horseradish peroxidase-conjugated second antibody (*C*) or autoradiography (*D*).

amino acids. TNFR-IgG was expressed in human embryonic kidney 293 cells by transient transfection with pRKTNFR-IgG by the calcium phosphate precipitation method as described (18). TNFR-IgG was purified to >95% homogeneity from serum-free cell culture supernatants by affinity chromatography on *Staphylococcus aureus* protein A. TNFR-IgG was eluted with 50 mM sodium citrate, pH 3/20% (wt/vol) glycerol, and the pH was neutralized with 0.05 vol of 3 M Tris·HCl (pH 8–9).

TNF Binding Assays. Binding of TNFR-IgG to TNF was analyzed essentially as described for CD4-IgG binding to HIV gp120 (20). TNFR-IgG (1 μg/ml) was immobilized onto microtiter wells coated with goat anti-human IgG Fc antibody. Reactions with recombinant human ^{125}I-labeled TNF-α (^{125}I-TNF-α; radioiodinated by using lactoperoxidase to a specific activity of 19.1 μCi/μg; 1 μCi = 37 kBq) were done in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 1 hr at 24°C. Nonspecific binding was determined by omitting TNFR-IgG. In competition binding analyses, ^{125}I-labeled TNF-α was incubated with immobilized TNFR-IgG in the presence of increasing concentrations of unlabeled TNF. The K_d was determined from competition IC_{50} values according to the following equation: $K_d = IC_{50}/(1 + [T]/K_{dT})$, where [T] is the concentration of the tracer (0.1 nM) and K_{dT} is the K_d of the tracer determined by saturation binding (80 pM).

TNF Cytotoxicity Assays. TNF cytotoxicity was assayed essentially as described (21). Murine L-M cells were plated in microtiter dishes (4×10^4 cells per well) and treated with actinomycin D (3 μg/ml) and TNF-α or TNF-β (1 ng/ml) in the absence or presence of TNFR-IgG or other inhibitors. After 20 hr of incubation at 39°C, the cell survival was determined by a crystal violet dye exclusion test.

Mouse Model for Septic Shock. Septic shock was modeled by endotoxin injection of 6- to 8-week-old female BALB/c mice. Animals were injected intravenously (i.v.) with an LD_{100} dose of *Salmonella abortus*-derived endotoxin (175 μg per mouse) in phosphate-buffered saline (PBS), and survival was followed for at least 78 hr. Purified TNFR-IgG, or CD4-IgG used as a negative control, were diluted in PBS and injected i.v. prior to or after endotoxin administration.

RESULTS

Subunit Structure of TNFR-IgG. TNFR-IgG was created by fusing complementary DNAs encoding the extracellular portion (amino acids 1–171) of human 55-kDa type 1 TNFR and the hinge region and constant region C_H2 and C_H3 domains (amino acids 216–443) of human IgG_1 heavy chain (Fig. 1*A*). A vector directing mammalian expression of TNFR-IgG was introduced transiently into human kidney 293 cells to produce the molecule as a secreted protein. Taking advantage of the presence of an IgG Fc domain in TNFR-IgG, we used protein A affinity chromatography to recover and purify the protein from cell culture supernatants. We examined the subunit structure of TNFR-IgG by SDS/polyacrylamide gel electrophoresis (Fig. 1 *B–D*). Under reducing conditions, a molecular mass of ≈60 kDa was observed, whereas under nonreducing conditions, it was approximately doubled, indicating that TNFR-IgG is a disulfide-bonded dimer (Fig. 1*B*). Minor bands of higher molecular mass were observed also, suggesting some aggregation of TNFR-IgG. Western blot analyses showed reactivity of TNFR-IgG with antibody to the type 1 human TNFR or to human IgG Fc (Fig. 1*C*) and showed specific binding of human ^{125}I-TNF-α (Fig. 1*D*). Notably, ^{125}I-TNF-α did not bind to reduced TNFR-IgG, suggesting that intramolecular disulfide bonds in TNFR are required for binding to TNF-α. These results indicate a covalent homodimeric structure for TNFR-IgG and the presence of functional TNF binding and antibody Fc domains in this protein.

Binding of TNFR-IgG to TNF-α and TNF-β. To investigate the binding of TNFR-IgG to TNF-α and TNF-β, we carried out saturation and competition binding analyses, using an assay in which TNFR-IgG was immobilized by binding of its Fc domain to anti-Fc antibodies coated on microtiter wells. Human ^{125}I-TNF-α bound to a single class of sites with an apparent dissociation constant (K_d) of 80 ± 20 pM (Fig. 2 *Left*). Human TNF-β was able to displace the binding of ^{125}I-TNF-α completely, confirming previous observations

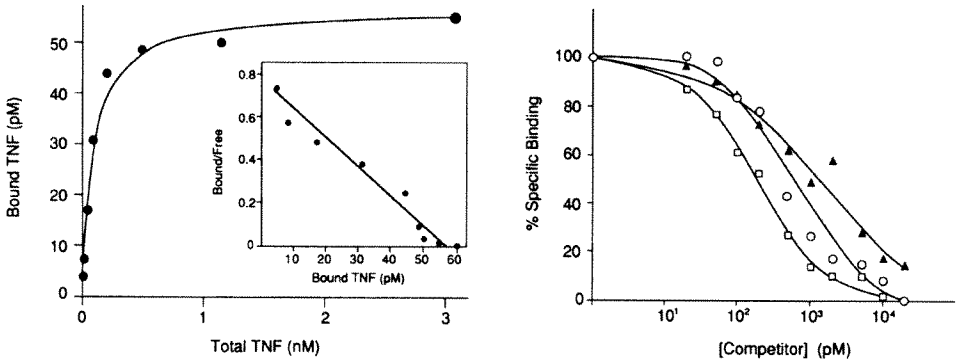

FIG. 2. Binding of TNFR-IgG to TNF-α and TNF-β. (*Left*) Saturation analysis of TNFR-IgG binding to human TNF-α. Purified TNFR-IgG was immobilized in microtiter wells coated with anti-IgG Fc antibody and incubated with increasing concentrations of recombinant human ^{125}I-TNF-α. Saturation and Scatchard (*Inset*) plots were generated by using the best fit as determined by unweighted least-squares regression analyses. (*Right*) Competition analysis of TNFR-IgG binding to recombinant human TNF-α (○), human TNF-β (▲), and murine TNF-α (□). TNFR-IgG was incubated with 100 pM ^{125}I-TNF-α in the presence of increasing concentrations of unlabeled competitor.

that both TNFs bind to type 1 TNFR (4–8). A K_d of 550 ± 100 pM was observed for TNF-β and a K_d of 75 ± 5 pM was observed for murine TNF-α (Fig. 2 *Right*). Notably, the K_d for binding of TNFR-IgG to TNF-α was significantly lower than values reported for type 1 cell-surface or soluble TNFR (sTNFR) (470–660 nM) (4–6). Thus, TNFR-IgG appears to bind to TNF-α with 6- to 8-fold higher affinity than type 1 cell surface TNFR or sTNFR. This higher affinity may be due to a multivalent interaction between TNFR-IgG and TNF-α, as the structure of TNFR-IgG is dimeric (Fig. 1) and the structure of TNF-α is trimeric (22–24). Indeed, saturation analysis in solution, in which complexes of ^{125}I-TNF-α and TNFR-IgG were precipitated quantitatively with protein A, showed a molar binding ratio of trimeric TNF-α and TNFR-IgG of 1.25 ± 0.05:1 (not shown). These results are consistent with the possibility that the two TNFR domains of a TNFR-IgG molecule interact with one TNF-α trimer, which may result in a more stable binding interaction. Alternatively, only one of two TNFR domains in TNFR-IgG may interact with a TNF-α trimer; this is less likely, however, since it would not be expected to result in higher binding affinity.

TNFR-IgG Blocks the Cytolytic Actions of TNF-α and TNF-β *in Vitro*. To test the ability of TNFR-IgG to antagonize TNF activity *in vitro*, we investigated the effect of TNFR-IgG on the induction of cell lysis by TNF-α in actinomycin D-treated murine L-M cells (Fig. 3 *Left*). While no inhibitory effect was observed with CD4-IgG, TNFR-IgG was able to block cell killing completely, with 50% inhibition (IC_{50}) occurring at 0.5 μg/ml (5 nM). For comparison, we tested type 1 sTNFR and found an IC_{50} of 3.5 μg/ml (120 nM). In addition, we tested the activity of two highly neutralizing monoclonal antibodies to human TNF-α (25) and found an IC_{50} of ≈3.5 μg/ml (≈21 nM). Thus, on a molar basis, TNFR-IgG was 24-fold more efficient than sTNFR and 4.2-fold more efficient than anti-TNF-α antibodies in blocking the cytolytic action of TNF-α. We tested also the ability of TNFR-IgG to block the cytolytic activity of TNF-β (Fig. 3 *Right*). Complete inhibition of cell killing was achievable, with an IC_{50} of 1.5 μg/ml (15 nM). Thus, TNFR-IgG was less efficient by a factor of 3 in blocking TNF-β than TNF-α, consistent with its lower affinity for TNF-β. These results show that TNFR-IgG acts as a full antagonist *in vitro* against both types of TNF.

TNFR-IgG Protects Against Septic Shock in Mice. To investigate the ability of TNFR-IgG to act as a TNF antagonist *in vivo*, we used a model for septic shock in mice (Fig. 4). In animals receiving an LD_{100} dose of endotoxin, complete lethality was observed within 48 hr. Injection of TNFR-IgG 0.5 hr prior to endotoxin administration prevented lethality at a TNFR-IgG dose of 20 μg per mouse and provided partial protection at lower doses, whereas CD4-IgG had no significant effect (Fig. 4 *Left*). We investigated the temporal relation of TNFR-IgG and endotoxin injection also (Fig. 4 *Right*). Injection of 10 μg of TNFR-IgG per mouse provided signif-

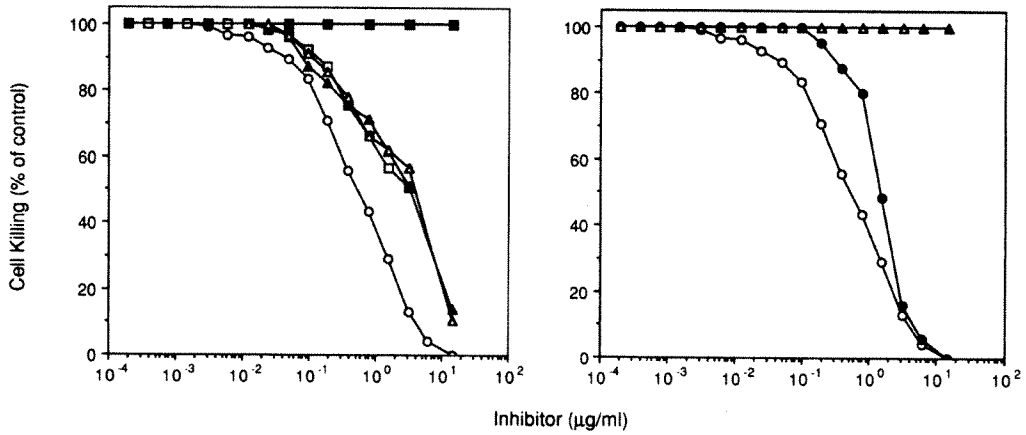

FIG. 3. Inhibition of TNF cytotoxicity by TNFR-IgG *in vitro*. (*Left*) Effect of TNFR-IgG (○), soluble type 1 TNFR (□), monoclonal antibodies D (△) or E (▲) to human TNF-α (15), or CD4-IgG (■) (11) on the killing of actinomycin D-treated murine L-M cells induced by TNF-α (1 ng/ml). (*Right*) Effect of TNFR-IgG (○, ●) or CD4-IgG (△, ▲) on cell killing by TNF-α (1 ng/ml) (○, △) or TNF-β (1 ng/ml) (●, ▲).

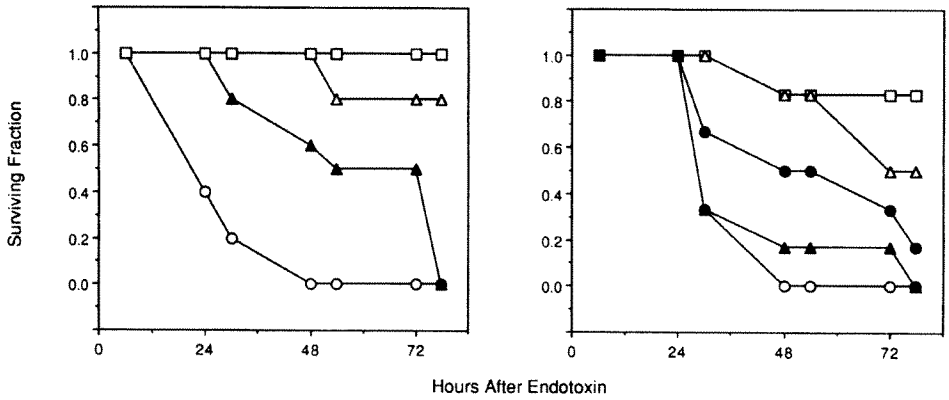

FIG. 4. Inhibition of endotoxin-induced lethality in mice by TNFR-IgG. (*Left*) Effect of the dose of TNFR-IgG on endotoxin-induced lethality. Mice (n = 10) were given an i.v. injection of TNFR-IgG at a dose per mouse of 20 μg (□), 4 μg (△), and 0.8 μg (▲) or CD4-IgG at 4 μg per mouse (○). Thirty minutes later, the mice were given an i.v. injection of endotoxin. Survival data is shown for the first 78 hr and remained unchanged for at least another week. (*Right*) Effect of the time of administration of TNFR-IgG in relation to the time of endotoxin challenge. Mice (n = 6) were given i.v. injection of endotoxin only (○) or of TNFR-IgG (10 μg per mouse) 0.5 hr before (□), 0.5 hr after (△), 1 hr after (●), or 2 hr after (▲) administration of endotoxin.

icant protection 0.5 hr before, 0.5 hr after, or 1 hr after endotoxin injection but little protection 2 hr after endotoxin injection. These data show that TNFR-IgG can prevent or significantly delay endotoxin-induced lethality in mice when given prior to or shortly after endotoxin challenge.

DISCUSSION

Our results show that TNFR-IgG, a molecule that combines the TNF binding function of the extracellular portion of type 1 TNFR with the dimeric structure of IgG, is a potent TNF antagonist. At the molecular level, TNFR-IgG exhibits significantly higher affinity for TNF-α than monomeric cell surface or soluble TNFR, and a molar binding ratio of 1.25:1 TNF-α trimer to TNFR-IgG suggests that this higher affinity may be due to bivalent binding to TNF-α.

At the cellular level, TNFR-IgG blocks the cytolytic action of TNF-α or TNF-β in murine L-M cells completely, and is markedly more potent than sTNFR or anti-TNF-α monoclonal antibodies in blocking TNF-α. The difference in TNF-α binding affinity between TNFR-IgG and sTNFR probably contributes to the differential efficiency of these forms of TNFR in blocking the TNF-α cytolytic activity. However, the difference between TNFR-IgG and sTNFR in blocking TNF-α (24-fold) is significantly greater than the difference in affinity (6- to 8-fold). Previous work with anti-TNFR antibodies showed that bivalent but not monovalent antibody fragments can activate TNFR (26), indicating that a TNF-α trimer may trigger signal transduction by cross-linking two cell surface TNFR molecules. Therefore, the ability of TNFR-IgG to block two receptor binding sites on a TNF-α trimer simultaneously, thus rendering TNF-α unable to dimerize cell surface receptors, also may contribute to the greater efficiency of TNFR-IgG vs. sTNFR in blocking TNF-α.

At the level of the whole organism, TNFR-IgG can prevent or protect against endotoxic shock in mice, depending on the dose and time of injection. This confirms the hypothesis that TNF-α is a key contributor to the septic shock syndrome, first suggested by the ability of anti-TNF-α antibodies to protect against septic shock (9, 10). The ability of TNFR-IgG to provide protection at the doses tested in this study appears limited to about 1 hr after endotoxin challenge. This is consistent with the finding that the rise in circulating levels of TNF-α in animals challenged with endotoxin or *E. coli* and in patients with septic shock is transient (13–17). Taken together, these observations support the notion that the transient increase in TNF-α following sepsis triggers a subsequent cascade of events that can lead to the pathogenesis of shock and multiple organ failure.

In the past few decades, major advances in the treatment of bacterial infections have been achieved, such as the development of powerful antimicrobial agents. Nonetheless, the number of cases with sepsis and the rate of mortality remain high (27). Recently, a monoclonal antibody to endotoxin, derived from human sources, has been shown to be partially protective in patients with septic shock (28). Another approach to the treatment of sepsis has been the administration of murine anti-TNF-α monoclonal antibodies (29). However, the use of murine antibodies in humans leads to the generation of anti-murine antibodies (29), which could hamper the action of the anti-TNF-α antibodies during repeated or chronic administration.

The observation that TNFR-IgG provides protection against endotoxin-induced lethality when given before and shortly after endotoxin administration suggests that this molecule may offer clinical potential both prophylactically in patients at high risk of sepsis and therapeutically in patients with shock. In contrast to murine anti-TNF-α antibodies, TNFR-IgG is derived from human proteins and therefore is expected to be much less immunogenic in humans, as indeed is the case for the similarly constructed CD4-IgG (A.A. and D.J.C., unpublished results). In addition, the increased affinity of TNFR-IgG for TNF-α appears to confer greater efficiency in blocking TNF-α *in vitro*, as compared with sTNFR or anti-TNF-α antibodies, although this remains to be investigated *in vivo*. Finally, since the rise in circulating TNF-α is subsequent to the occurrence of endotoxemia, it may be possible to extend the time window for treatment of septic shock by combination therapy with anti-endotoxin antibodies and anti-TNF-α agents such as TNFR-IgG.

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Attenuation of Collagen-Induced Arthritis in 55-kDa TNF Receptor Type 1 (TNFR1)-IgG1-Treated and TNFR1-Deficient Mice

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The role of TNF and its type 1 receptor (TNFR1) in the pathogenesis of collagen-induced arthritis (CIA) was investigated in mice using two approaches. First, DBA/1 mice were treated after immunization with type II collagen by injecting TNFR1-IgG1 fusion protein to neutralize systemic TNF. CIA was prevented when treatment was administered shortly before the onset of clinical disease, suggesting that TNF is a crucial mediator in the late initiation phase of the arthritic process. In a second approach, TNFR1-deficient mice, generated by gene targeting and crossed to DBA/1, were used. These mice developed CIA with a low incidence and in a milder form. However, once a joint was afflicted, the disease progressed in this joint to the same end stage as that in wild-type mice. These data suggest that TNFR1 is the main transducer of TNF proinflammatory effects establishing CIA, but the progression of arthritis to tissue destruction and ankylosis is independent of TNFR1. ***The Journal of Immunology,*** **1996, 157: 3178–3182.**

Collagen-induced arthritis (CIA)[3] in mice is an experimental autoimmune disease model that is thought to reflect aspects of human rheumatoid arthritis. CIA develops in mice after immunization with native type II collagen (CII) in adjuvant (1). The disease is mediated by autoreactive T lymphocytes (2), and specific H-2 alleles (3–5), TCR β genes (6–8), as well as other unknown elements in the genetic background confer susceptibility (8). The induction of arthritis absolutely requires a sustained inflammatory response (9). The role of the proinflammatory mediator TNF in CIA has been previously investigated in attempts to understand the pathogenic mechanisms and to identify targets for rational therapeutic intervention (10–13). Such studies have led to the view that TNF, in the context of other cytokines, has an important role in the initiation, maintenance, and progression of local inflammatory responses and tissue injury in arthritis (reviewed in Ref. 14). The pathogenic role of the proinflammatory cytokines in arthritis is further supported by studies conducted with mice carrying a human TNF-α transgene; as a consequence of constitutive and deregulated TNF expression, these animals develop a chronic inflammatory polyarthritis that can be prevented by anti-TNF mAb, even though human TNF is not detected in systemic circulation (15), and by anti-IL-1R type I Ab treatment (16). The importance of the tissue distribution of deregulated TNF production, suggesting a paracrine rather than systemic type of activity, is further supported by another study, in which transgenic mice with a TNF gene construct under control of a heterologous tissue-specific promoter developed a number of TNF-dependent lesions, but no arthritis (17).

The activities of TNF are mediated by two distinct cellular TNF receptors, TNFR1 (CD120a) and TNFR2 (CD120b) (for review, see Ref. 18). Soluble extracellular domains of these receptors with TNF binding activity naturally occur in plasma by proteolytic cleavage of the membrane receptors. Depending on their distinct TNF binding properties, these two soluble receptors may function as TNF inhibitors or as a slow release reservoir (19–21). Recombinant analogues of the soluble TNF receptors and recombinant chimeric proteins combining the TNFR extracellular domain with IgG heavy chain fragments showed potent TNF-neutralizing activity in in vitro studies (22, 23) and in vivo using various disease models, including CIA (11, 12, 24–27).

In the present study we investigated the effect of functional TNF inhibition on the appearance and evolution of CIA using a recombinant chimeric soluble TNFR1-IgG1 protein construct and TNFR1-deficient mice with a CIA-susceptible genetic background. The study of the development and progression of the disease in these mice demonstrated that TNF has an important role in the late initiation phase of the arthritic process, and that TNFR1 is responsible for most, but not all, the pathogenic effects in CIA.

Materials and Methods

Mice

DBA/1J mice were obtained from Biologic Research Laboratories (Fullinsdorf, Switzerland) and maintained in the mouse colony at Hoffmann-La Roche (Basel, Switzerland). TNFR1-deficient mice were generated by gene targeting (28). Disruption of the Tnfr1 gene was achieved by homologous recombination with a gene-targeting vector in which exon 2, exon 3, and part of exon 4 of the mouse Tnfr1 gene were replaced by a neomycin cassette. Chimeric mice were generated by microinjection of E14 ES cells (129 mouse strain-derived) carrying a mutated Tnfr1 allele into C57BL/6 host blastocysts. Germ-line transmitters of the mutated Tnfr1 allele were crossed with C57BL/6 mice, and the resulting heterozygous mice were interbred to yield homozygous mutant offspring. Homozygous Tnfr1°/Tnfr1°C57BL/6 × 129 mice were crossed twice to DBA/1J mice to introduce CIA susceptibility genes. Hybrid mice were selected according to the

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[3] Abbreviations used in this paper: CIA, collagen-induced arthritis; CII, collagen type II; TNFR1, 55-kilodalton TNF receptor type 1; TNFR2, 75-kilodalton tumor necrosis factor receptor type 2; TNFR1-IgG1, human recombinant soluble tumor necrosis factor receptor type 1-immunoglobulin G1-fusion protein Ro 45-2081; Tnfr1°/Tnfr1°, tumor necrosis factor receptor type 1-deficient mice; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin.

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following criteria: 1) homozygosity at the H-2^q class II locus, 2) heterozygosity at the Tnfr1 locus (Tnfr1°/Tnfr1$^+$), and 3) sensitivity to CIA. Mice meeting all three criteria were further interbred to generate homozygous Tnfr1°/Tnfr1°-H-2^q as well as heterozygous Tnfr1$^+$/Tnfr1°-homozygous H-2^q littermate mice. Mice were screened for Tnfr1 genotype by PCR using DNA prepared from tail biopsies as previously described (29). PCR was performed using the following two sets of primers to distinguish between homozygous and heterozygous TNFR1 knockout and wild-type mice: 5′-TCCC GCTTCAGTGACAACGTC and 5′-GTTAGGTCTCTCCTGAATGTGATC, and 5′-CTCTCTTGTGATCAGCACTG and 5′-CTGGAAGTGTGTCTCAC. Using the first set of primers, only DNA from mice with at least one copy of the mutated TNFR1 gene produced an amplified fragment of approximately 2 kb. Using the second set of primers, amplified DNA fragments were about 1.4 kb for wild-type mice, about 2 kb for homozygous TNFR1 knockout, and about 2 and 1.4 kb for heterozygous mice.

Induction and evaluation of CIA

Mice (8–11 wk old) were randomly divided into groups and immunized by intradermal injection in the back at the base of the tail with 200 μg of bovine CII in CFA (Difco, Detroit, MI). CII was kindly provided by Dr. D. Bradshaw (Roche Products, Welwyn Garden City, U.K.). An i.p. injection of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG; Schweiz. Serum & Impfinstitut, Bern, Switzerland) was given to the mice at the same time, as previously reported (8). Twenty-one days after the immunization, mice were boosted i.p. with 200 μg of CII and BCG unless they had already developed arthritis. Arthritis was monitored by visual inspection for up to 3 mo, unless otherwise stated, according to the following schedule: daily from days 15 to 18 until day 36, every third day during an additional month, and every second week thereafter. The dates of onset of disease were recorded for all mice. CIA was diagnosed by the clinical criteria of redness and swelling, deformity, and ankylosis of fore and hind paws. The severity of arthritis was scored for each paw and graded: grade 0, normal appearance and flexion; grade 1, erythema and edema; grade 2, deformity; and grade 3, ankylosis detectable on flexion. The scores for each paw of a given individual animal were added to give an arthritis score ranging from 0 to 12/mouse. Mice only injected with CFA and BCG never developed CIA, as previously reported (8). Statistical analysis was performed using the χ^2 test for comparing CIA incidence and the Mann-Whitney test for comparing CIA severity and day of onset between the experimental groups.

Histopathology

At the end of the experiments, i.e., 3 mo after disease induction, arthritic and control paws (one or two per mouse) were removed post mortem, fixed for >48 h in 10% neutral formalin, and gently decalcified for 14 days under constant moving in EDTA/phosphate buffer, pH 7.4. Paws were then cut sagitally in half and embedded in paraffin; five or six serial sections were obtained and stained with hematoxylin-eosin (four or five sections) and Van Gieson (one section). Microscopic evaluation was performed in a single blind manner. The histologic severity of arthritis was classified as mild, moderate, or severe according to the criteria reported by Williams et al. (10). Each joint articulation in the limbs was evaluated by analyzing all serial slides, and an average histologic grade was given.

Treatment protocols with TNFR1-IgG1

The soluble TNFR1-IgG1 fusion protein (Ro 45-2081) was constructed by fusing the extracellular domain of the human TNFR1 cDNA to a human IgG1 heavy chain gene fragment containing the hinge, CH2, and CH3 domains (24). The recombinant fusion protein, in a buffer containing 10 mM citric acid, 23 mM glycine, and 230 mM mannitol, pH 6.0 (Ro 45-2081/003), was produced at Genentech (South San Francisco, CA; courtesy of Dr. S. Chamow) and formulated by Dr. A. Ferro (F. Hoffmann-La Roche). In the single dose studies, mice were randomly assigned to the various treatment groups before CII immunization; those animals assigned to a group that was to receive late treatment (i.e., treatment on days 21 and 28) and that had already developed signs of disease on the scheduled treatment day were disregarded for this analysis. Two hundred micrograms of TNFR1-IgG1/mouse was injected i.p. in a volume of 200 μl into CII-immunized male mice on day 7, 14, 21, or 28 after the start of the immunization. In the first part of the multiple dose study, female mice were injected i.v. into the orbital venous plexus (200 μl) every third day, starting from day 18 or day 21, until day 42 after immunization with CII. The initial dose was 200 μg of TNFR1-IgG1/mouse, followed every third day with 144 μg/mouse. In the second part of the multiple dose study, male mice were injected i.v. every third day from day 18 until day 36 after immunization with the doses described above. In the third part of the multiple dose study, daily i.p. injections of 50 μg/mouse in 200 μl of buffer were performed for 8 days from day 21 until day 28 after immunization according to the protocol of administration of a TNFR2-IgG1 construct reported by Wooley et al. (11). Control mice for each experiment were injected with placebo carrier buffer according to the various treatment schedules.

Serum concentrations of TNFR1-IgG1 and anti-TNFR1-IgG1 antibodies

Blood samples of ~200 μl were collected from the orbital plexus 20 days after the first TNFR1-IgG1 injection and 18 days after the end of the treatment (i.e., day 60 after CII immunization). TNFR1-IgG1 concentrations in serum were detected using an ELISA assay, as described by Gallati and Pracht (30). Briefly, sera were incubated on microtiter plates coated with mAb htr-20 specific for TNFR1, and specifically bound material was revealed using peroxidase-labeled human rTNF-α. Anti-TNFR1-IgG1 Abs were detected using an ELISA, as described by Gallati and Pracht (31). Briefly, sera were incubated on microtiter plates coated with TNFR1-IgG1, and specifically bound material was revealed using peroxidase-labeled TNFR1-IgG1.

Results

Critical role of TNF late in the initial phase of CIA

CIA in DBA/1 mice develops in clearly recognizable disease phases. The initial phase, from the immunization with CII until the appearance of the first clinical arthritic manifestations, has highly variable lengths in individual animals, usually lasting 18 to 36 days from the immunization. In the second phase, starting with clinically apparent disease onset, paw erythema and edema appear; these signs correlate with inflammatory tissue insult, which may be transient, but in many cases, swelling of the affected joints persists. These alterations are prodromic for the third phase of the disease when ankylosis of the articulations occurs; this may affect all four paws in the most severe forms. The progression to the final stage takes up to about 70 days after the immunization.

In a preliminary study, to investigate to which extent and at which time point of the first disease phase TNF is critically involved in the establishment of CIA, DBA/1 mice were injected with a single high dose of TNFR1-IgG1 at different times after immunization with CII, but before the development of clinical signs of CIA. These results suggested that a single high dose of TNFR1-IgG1 given in the late phase of disease initiation (i.e., on day 21 or day 28) has a preventive effect, while it has no effect when given on day 7 or day 14 after immunization (data not shown).

To test whether full protection could be achieved by a more complete TNF neutralization, treatment protocols with multiple administrations of TNFR1-IgG1 were investigated in further studies. Mice were injected i.v. with multiple doses, calculated to maintain an estimated average steady state plasma concentration of TNFR1-IgG1 of about 50 μg/ml. These high doses and route of administration were chosen in an attempt to induce tolerance to the human TNFR1-IgG1 construct and, thus, to avoid production of Abs that would lead to neutralization and rapid elimination of the drug. According to these treatment protocols, mice were injected with TNFR1-IgG1 every third day, starting from day 18 or day 21 after the immunization and continuing until day 36 or 42, to cover the entire time period during which the first clinical signs of CIA appear (Table I). Groups of female mice immunized with CII received repeated injections of placebo or TNFR1-IgG1 starting on day 18 or day 21. Both active treatment groups showed a tendency for reduced CIA incidence (1 of 10 and 2 of 10 animals, respectively) and severity (arthritis score 3) compared with placebo control mice (4 of 10 animals, with median arthritis score 5.5; Table I). In an additional series of studies, male mice were investigated. CII-immunized mice were treated every third day from days 18 to 36 according to the above protocol; a complete inhibition of clinical signs of CIA was observed (incidence 0), compared with parallel placebo control mice, which developed CIA with an incidence

Table I. *Effect of multiple TNFR1-IgG1 administrations on CIA prevention in DBA/1 mice*

Mice	Treatment	Arthritic/Total Number of Mice		Incidence %	Day of Onset Median (range)	Severity Median (range)
Female	Placebo	4/10		40	29.5 (29–33)	5.5 (3–12)
	Day 18–42 (i.v.)	1/10	($p < 0.2$)	10	33	3
	Day 21–42 (i.v.)	2/10	($p < 0.4$)	20	37.5 (33–42)	3
Male[a]	Placebo	7/10		70	17 (17–27)	4 (1–12)
	Day 18–36 (i.v.)	0/9	($p < 0.002$)	0		0
	Day 21–28 (i.p.)	0/10	($p < 0.001$)	0		0

[a] Mice were scored at day 76.

Table II. *CIA in TNFR1 knockout/H-2^q mice*

Mice	Arthritic/Total[a]	Incidence[b]	Severity[c]	Affected Limbs[d]	Onset Day[e]
Homozygous Tnfr1°/Tnfr1° (TNFR1 −)	6/33	18.2%	3 (3–9)	1 (1–3)	28 (20–78)
Heterozygous Tnfr1°/Tnfr1$^+$ (TNFR1 +)	9/14	64.3%	9 (6–12)	3 (2–4)	24 (15–32)

[a] Number of mice from three experiments using both female and male animals.
[b] $p < 0.002$.
[c] Median value (range), $p < 0.02$.
[d] Median value (range), $p < 0.03$.
[e] Median value (range).

of 70% and a median arthritis score of 4 (Table I), demonstrating a higher efficacy of treatment than in female mice.

The serum TNFR1-IgG1 concentration and the Ab response to the injected heterologous recombinant protein were measured in two serum samples taken from each animal during (i.e., after the seventh dose) and 18 days after the end of treatment. TNFR1-IgG1 was present at high levels (mean ± SD, 74.5 ± 49.2 μg/ml) during treatment and could still be detected 18 days after the end of treatment (0.29 ± 0.12 μg/ml). At these same time points, no anti-TNFR1-IgG1 Abs were detected in the serum, demonstrating the tolerant state of these mice to the human receptor construct (data not shown).

To further explore the influence of the administration protocol on the treatment efficacy, male mice were injected i.p. daily with TNFR1-IgG1 from days 21 to 28 after the CII immunization. A similar dosing schedule had previously been reported in an independent CIA study (11). None of the 10 mice treated according to this protocol developed CIA, whereas 7 of 10 mice in the parallel placebo control group developed disease of median score 4 (Table I). In conclusion, these findings show that systemic neutralization of TNF by TNFR1-IgG1 treatment shortly before or concomitantly with clinical disease onset is sufficient for CIA prevention, thus demonstrating the critical role of TNF in the induction phase of arthritis.

CIA is attenuated in TNFR1-deficient mice

After systemic administration, TNF-neutralizing compound TNFR1-IgG1 essentially distributes into the plasma compartment (32). To elucidate whether neutralization of TNF activity throughout tissues will also result in the complete control of CIA, the disease was studied in TNFR1 knockout mice, because this receptor is thought to mediate the majority of TNF activities (for review, see Ref. 33). These mice were bred into a CIA-susceptible background by crossing C57BL/6 × 129 Tnfr1°/Tnfr1° mice (28) to DBA/1 mice (see *Materials and Methods*). Homozygous Tnfr1°/Tnfr1°-H-2^q and heterozygous Tnfr1$^+$/Tnfr1°-H-2^q mice were immunized with CII and scored for the development of CIA. The incidence and the severity of the disease in Tnfr1$^+$/Tnfr1°-H-2^q mice were similar to those in DBA/1 mice (data not shown). However, Tnfr1°/Tnfr1°-H-2^q mice showed a milder form of disease; in these animals, disease incidence was reduced from 64.3 to 18.2%, and severity was reduced from a median arthritic score of 9 to a score of 3 compared with that in the control Tnfr1$^+$/Tnfr1°-H-2^q heterozygous littermates (Table II). No significant differences were observed between male and female knockout mice (data not shown). The time of disease onset in the homozygous mice was not significantly different from that in heterozygous littermates (Table II). In most of the TNFR1 knockout mice, only one limb was affected, which accounts for the reduced severity score (Table II). However, in those joints of the mutant animals that became afflicted, the disease progressed to the most severe end stage with the same clinical and histologic characteristics as those in the heterozygous control littermates. Histologic evaluations confirmed the characteristic arthritic lesions and showed an excellent correlation with clinical grading. The histology of afflicted joints of both normal and TNFR1-deficient mice showed severe arthritis characterized by chronic inflammatory infiltration of the synovia, extensive cartilage loss, and bone erosion, leading to disrupted and sometimes ankylosed joint architecture (Fig. 1). Thus, in mice lacking TNFR1, CIA developed with reduced incidence. The disease severity was also reduced as a consequence of the fact that in those knockout mice that did develop the disease, a smaller number of joints was involved. However, once the arthritic process was initiated, the lesions progressed to the same extent in TNFR1-deficient as in wild-type animals. These findings confirm the importance of TNF in mediating CIA and show that TNFR1 is the major, but not the only, promoter of disease-inducing mechanisms.

Discussion

This study assessed the pathogenic role of TNF in CIA by two different approaches. In the first approach, TNF was neutralized by treating CII-immunized mice with TNFR1-IgG1 according to various dosing schedules. TNFR1-IgG1, when injected i.v. or i.p., distributes practically exclusively in the central compartment, effectively neutralizing systemic TNF; however, it is less well understood to what extent it interferes with TNF activity in tissues (32). All the different administration protocols that were tested demonstrated the efficacy of systemic TNFR1-IgG1 treatment in

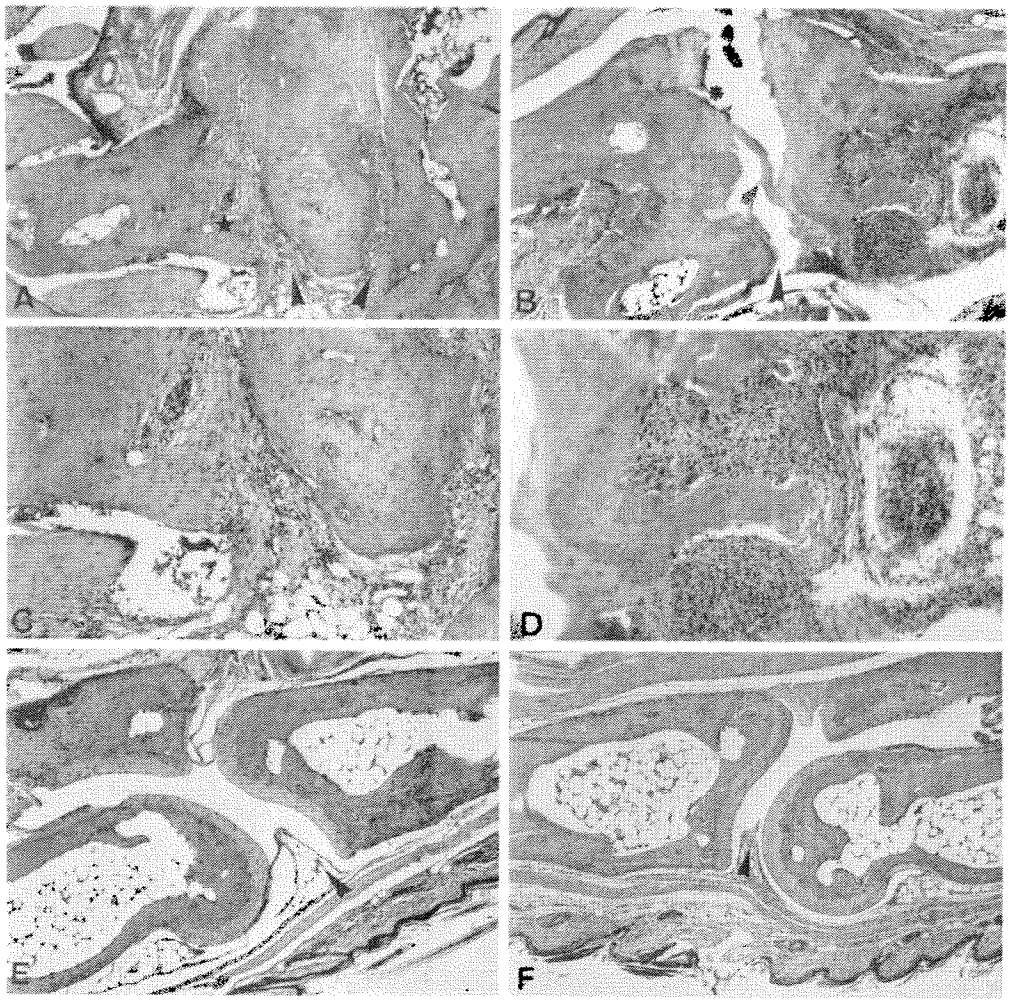

FIGURE 1. Histopathological evaluation of severe arthritis in both TNFR1-deficient and sufficient mice. *A* and *C* represent a section of proximal articulation (intratarsal) of a hind limb from a homozygous Tnfr1°/Tnfr1°-H-2^q mouse. In *A* (hematoxylin-eosin stain; original magnification, ×80), ankylosis and marked bone erosion (★) are detectable; at higher magnification (*C*; ×160), moderate chronic synovialitis is clearly seen. *B* and *D* represent a section of distal articulation (interphalangeal) of a hind limb from a Tnfr1$^+$/Tnfr1°-H-2^q mouse. In *B* (hematoxylin-eosin stain; original magnification, ×80), severe arthritis with marked intra-articular cartilage loss (*) and bone erosion are detectable; at higher magnification (*D*; ×160), bone erosion with chronic cellular infiltrate is clearly seen. *E* and *F* represent sections of control nonarthritic joints (metatarsophalangeal) of Tnfr1°/Tnfr1°-H-2^q and Tnfr1$^+$/Tnfr1°-H-2^q mice, respectively (hematoxylin-eosin stain; original magnification, ×80). Arrowheads indicate the joint spaces.

long term prevention of CIA when given shortly before or concomitantly with clinical disease onset. Indeed, the disease could be completely prevented when the treatment was applied for the minimal time interval from days 21 to 28 after immunization. The ability to prevent CIA by systemic TNF neutralization in the late days after the immunization might be due to the fact that TNF has a critical role in the initiation of the inflammatory phase occurring after recruitment and expansion of anti-collagen lymphocytes. These results confirm and extend those of previous studies, documenting that TNF plays a critical role in initiating the immunoinflammatory cascade leading to clinical manifestation of arthritis (10–14, 34). In one of these reports, neutralizing anti-TNF Abs, given in two injections 14 and 21 days after CII immunization, or a recombinant soluble TNFR1, continuously administered with an osmotic minipump for 15 days, significantly reduced CIA severity (12). However, this study did not address the effect on disease incidence. In another study, anti-TNF Abs, injected on days 14 and 18, delayed the development of CIA without affecting disease incidence or terminal severity (13). In a third study, neutralizing anti-TNF mAbs reduced CIA severity, as determined by paw swelling and histopathology. This treatment protocol, consisting of four weekly injections starting the day before immunization, did not affect the time of onset or the incidence of arthritis (10). A recombinant TNFR2-IgG1 soluble protein was also found to have preventive effects on CIA with regards to both the incidence and the clinical arthritic index (11). The combined evidence of our results and these reports demonstrate that TNF has a critical role in the development of CIA in the time window from the late period of the initial phase after immunization until the time when the first clinical manifestations appear. Differences in experimental design and in specific pharmacokinetic and TNF binding properties of the various TNF-neutralizing compounds might explain the observed variations in the efficacies of treatments (32).

The second part of the present study, addressing CIA in TNFR1 knockout mice, focused on the role of TNF throughout the tissue and on whether its signaling through TNFR1 is necessary for the

pathogenesis of CIA. Although TNFR1-deficient mice express functional TNFR2, there is broad evidence that TNFR1 is the dominant receptor mediating TNF activities (33). Supporting the view of a critical role of TNF in CIA development, the Tnfr1°/Tnfr1°-H-2^q mice had dramatically reduced disease incidence and severity. However, 18% of these mice still developed CIA. The number of afflicted joints was smaller, leading to a lower severity score value, but once the disease was established in a given joint, it progressed in severity in a fashion similar to that seen in heterozygous and wild-type mice. Histologic comparison of the joints from both normal and TNFR1 mutant animals showed similar types of lesions, suggesting that once the inflammatory reaction has initiated the destruction of cartilage and the progressive erosion of bone, the absence of a functional TNFR1 does not influence the chronic evolution of the disease. The present data do not rule out entirely that CIA in TNFR1-deficient mice might be sustained by TNFR2 signaling, but TNFR1 appears to have the dominant, although not exclusive, control in the initiation of the disease, whereas the progression to tissue lesions leading to ankylosis also appears in the absence of TNFR1. This observation is consistent with the studies demonstrating important functions of other cytokines in CIA. Indeed, the involvement of IFN-γ in the induction phase of CIA has recently been reported (35). The interactive nature and the redundancy of the cytokine network are further demonstrated in a recent study on the role of IL-1 in arthritis development in TNF transgenic mice, possibly through positive feedback mechanisms (16).

Despite these complexities, TNF and TNFR1 have predominant roles in the establishment of CIA, suggesting that tight control of TNF discloses an important therapeutic approach for chronic inflammatory diseases of autoimmune origin.

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Evolution of collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor

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SUMMARY

Immunization of DBA/1 mice with type II collagen within complete Freund's adjuvant leads to arthritis, lasting more than 3 months. Injection of anti-tumour necrosis factor (TNF) IgG, 2 and 3 weeks after immunization prevented the development of arthritis in the following months. This treatment had no effect when started 2 months after induction of the disease. A soluble form of the human recombinant TNF receptor type-β (rsTNFR-β), continuously infused at a rate of 20 μg/day during the second and third week after immunization, also had a long-term protective effect. Anti-TNF antibody had no effect upon the production of anti-type II collagen antibodies. These results indicate that TNF is critically involved in an early phase of this arthritis.

INTRODUCTION

Immunization of rodents with articular or type II collagen can elicit polyarthritis with analogies to rheumatoid arthritis. Susceptibility is genetically determined, mice bearing the H-2^q haplotype being highly susceptible.[1] This haplotype is apparently required for the response of T lymphocytes to type II collagen and indeed, depletion of $CD4^+$ T-lymphocyte subset prevents the development of arthritis.[2] Tumour necrosis factor (TNF) is one of the cytokines involved in T-cell-induced immunopathological reactions,[3,4] which are detected in the synovial fluid of various types of arthritis in human.[5] Some of its properties observed *in vitro*, such as the growth-promoting activity for fibroblasts,[6] the capacity to induce bone resorption,[7,8] or the secretion of other cytokines, such as interleukin-1 (IL-1),[9,10] granulocyte–macrophage colony-stimulating factor (GM-CSF)[11] or IL-6,[12] might be relevant to the manifestations of the disease.

The production of cytokines during the collagen arthritis model in rodents has been little explored, due to difficulties of obtaining joint fluid. However, it is possible to influence the course of the disease by the injection of cytokines or cytokine antagonists. Thus the administration of anti-interferon-γ (IFN-γ) antibody has been shown to influence the evolution of the adjuvant arthritis, with various effects depending upon the time of administration.[13]

In this report, we explored the role of TNF in the collagen arthritis by: (1) an evaluation of the TNF mRNA level in the footpad, and (2) a modulation of the disease by treatment with two types of TNF antagonists, rabbit anti-mouse TNF-α IgG, as used in previous studies,[4] or a new class of antagonists, the recombinant soluble human TNF receptor (rsTNFR). Both types of antagonists were capable of blocking evolution of arthritis.

MATERIALS AND METHODS

Mice

DBA/1 (H-2^q) mice were purchased from Olac Ltd (Bicester, U.K.) and were bred for less than five generations in our animal house.

Collagen arthritis

Male DBA/1, 2–3 months old, were injected with 0·1 ml of an emulsion made of complete Freund's adjuvant (CFA) and saline containing 100 μg of chicken type II collagen (Genzyme, Cambridge, MA) in the root of the tail. In some experiments, a second injection of collagen in CFA was given after 2 weeks. Footpad thickness was measured at various times, using a micrometer (Oditest, HC Kroeplin, GMBH, Schluechtern Germany).

RNA analysis

Immediately after death by exsanguination, the footpads were severed and frozen in liquid nitrogen. They were subsequently homogenized in guanidine thiocyanate, and the RNA were isolated by guanidine–thiocyanate/Cesium chloride centrifuga-

Abbreviations: rsTNFR-β, recombinant soluble extracellular region of the human TNF receptor type-β; TNF, tumour necrosis factor.

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tion.[14] Northern blots were prepared as described elsewhere, using ^{32}P-labelled mRNA obtained from a sP64 plasmid containing the 696 Taq-*Eco*RI fragment of the mouse TNF gene as a probe.[14] After hybridization, the filters were exposed for up to 7 days. The loading of the gels was evaluated by a staining of the membranes with methylene blue and also by hybridization with a 1·1 kilobase (kb) chicken muscle GAPDH cDNA,[15] subcloned into the *Pst*I site of pSP64. GAPDH was linearized with *Eco*RI and used as a template for bacteriophage SP6 RNA polymerase.

Anti-TNF antibody

Mouse *Escherichia coli* recombinant TNF-α (rTNF) was a kind gift from B. Allet (Glaxo IMB, Geneva, Switzerland). Antibodies were prepared in rabbits, by the repeated injections of cytokines in CFA, as previously described.[16,17] The IgG fractions were ultracentrifuged (150,000 *g* for 120 min) before injection in order to reduce their immunogenicity. Approximately 10 μg of the IgG fraction neutralized 1 μg of the recombinant cytokine in the TNF bioassay.[18]

Construction, expression and purification of a soluble TNF receptor

The cDNA encoding the extracellular domain of the human 55,000 MW TNF receptor (rsTNFR-β) was expressed in CHO$^-$ cells and the protein was purified by TNF-α affinity chromatography and gel filtration.[19,20] The rsTNFR-β, at a concentration of 1·5 mg/ml, or its solvent as a control, were introduced into Alzet osmotic minipumps (no. 2002, Alza, Palo Alto, CA), implanted intraperitoneally. As detected by the limulus amoebocyte lysate assay, the lipopolysaccharide (LPS) contamination was <50 ng/mg of protein. The rate of delivery was approximately 20 μg of protein/day for 15 days.

Assay of anti-type II collagen antibody

Collagen extracted from the rat xyphoid (a kind gift from A. Grimaud, Institut Pasteur, Lyon, France) was used in an ELISA. Briefly, microtitre plates were coated with collagen (10 μg/ml) in carbonate buffer 0·01 M pH 8·5. Dilution (1/100) of serum made in phosphate-buffered saline (PBS)+1% bovine serum albumin (BSA) and 0·05% Tween 20 were incubated overnight. The presence of mouse IgG was revealed with alkaline phosphatase-labelled goat anti-mouse IgG (Cappel Laboratories, Zürich, Switzerland). Plates were read in a spectrophotometer at 405 nm.

Histology

Footpads were fixed in Bouin's solution and embedded within paraffin. Sections were stained with haematoxylin and eosin (H & E).

Statistical evaluations

Means were compared using non-parametric Mann–Whitney *U*-test.

RESULTS

Expression of TNF mRNA

TNF mRNA was detectable at a low level (i.e. after an exposition of the blots of 7 days) in the footpad RNA isolated from untreated control mice (Fig. 1). Among the RNA isolated 4 weeks after immunization with type II collagen, some exhibited a higher TNF mRNA level than that observed in untreated mice (Fig. 1). This heterogeneity was not due to an inequality of the loading of the RNA, as shown by methylene blue staining or hybridization with a 'house-keeping' gene, GAPDH (not shown), but corresponded rather to variations in the response of individual mice. The TNF mRNA levels detected in the footpad 3 months after immunization were similar to those of untreated mice (Fig. 1).

Effect of anti-TNF IgG

Immunization with collagen in CFA led to chronic arthritis, as evidenced by an increase in footpad thickness; this became manifest after 1 month and slowly increased for up to 2–3 months after immunization.[21] Treatment with two injections of 1·5 mg of anti-TNF IgG, on the second and third week after immunization (i.e. before the development of arthritis), completely prevented further evolution of the disease (Table 1). Thus, 3 months after the first immunization and 2 months after the last injection of antibody, the difference in the footpad thickness of mice injected with non-immune or anti-TNF IgG was still highly significant (Table 1). This treatment prevented the various components of arthritis seen on histological sections, such as new bone formation, mainly responsible for the enlargement of the footpad, as well as inflammation of the synovia (Fig. 2). A more sustained treatment, but started at 2 months, i.e. when the arthritis was fully established, had no detectable effect (Table 1).

Effect of the rsTNFR-β

The rsTNFR-β was administered at the time when anti-TNF antibody was found to be effective. Continuous infusion of rsTNFR-β had a blocking effect upon the evolution of arthritis, comparable to that of anti-TNF antibody (Table 1).

Effect of anti-TNF antibody upon anti-collagen antibodies production

Administration of anti-TNF antibody had no detectable influence upon the serum titre of anti-collagen antibodies (Table 2).

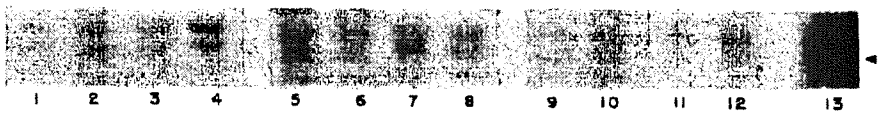

Figure 1. Expression of TNF in the hind footpad RNA. Northern blots of the foot pad RNA of normal mice, (1–4) or mice immunized with collagen and killed 4 (5–8) and 12 (9–12) weeks after immunization. As a positive control (13), the RNA from the lung of a mouse injected with LPS 1 hr before death.

Table 1. Treatment of collagen arthritis with anti-TNF agents

Immunization*	Treatment†	Weeks	Footpad thickness 1 month	Footpad thickness 3 months	
None	None	—	163 ± 6	163 ± 7	
CFA	None	—	169 ± 10	174 ± 8	
CII + CFA (1)	Normal IgG	2 and 3	178 ± 6	180 ± 10	
CII + CFA	Anti-TNF IgG	2 and 3	161 ± 13	160 ± 10	$P < 0.001$
CII + CFA (2)	Normal IgG	2 and 3	175 ± 13	220 ± 22	
CII + CFA	Anti-TNF IgG	2 and 3	168 ± 6	160 ± 10	$P < 0.0001$
CII + CFA (2)	Normal IgG	> 8	—	181 ± 5	
CII + CFA	Anti-TNF IgG	> 8	—	182 ± 9	
CII + CFA (1)	Solvent	2 and 3	189 ± 11	195 ± 11	
CII + CFA	rsTNFR-β	2 and 3	176 ± 10	182 ± 18	$P < 0.01$

Results are the mean ± SD of the footpad thickness in mm × 10^{-2} from six or more mice.

* Mice were immunized once (1) or twice (2) with type II collagen as described in Materials and Methods.

† 1·5 mg of anti-cytokine IgG was injected i.p. in two injections, the second and third week after immunization or in weekly injections, starting 8 weeks after immunization. Soluble TNF receptor or its solvent were delivered for 15 days by an osmotic minipump, implanted i.p. on Day 15 after immunization. Difference with the control groups, treated with non-immune IgG or the solvent are indicated.

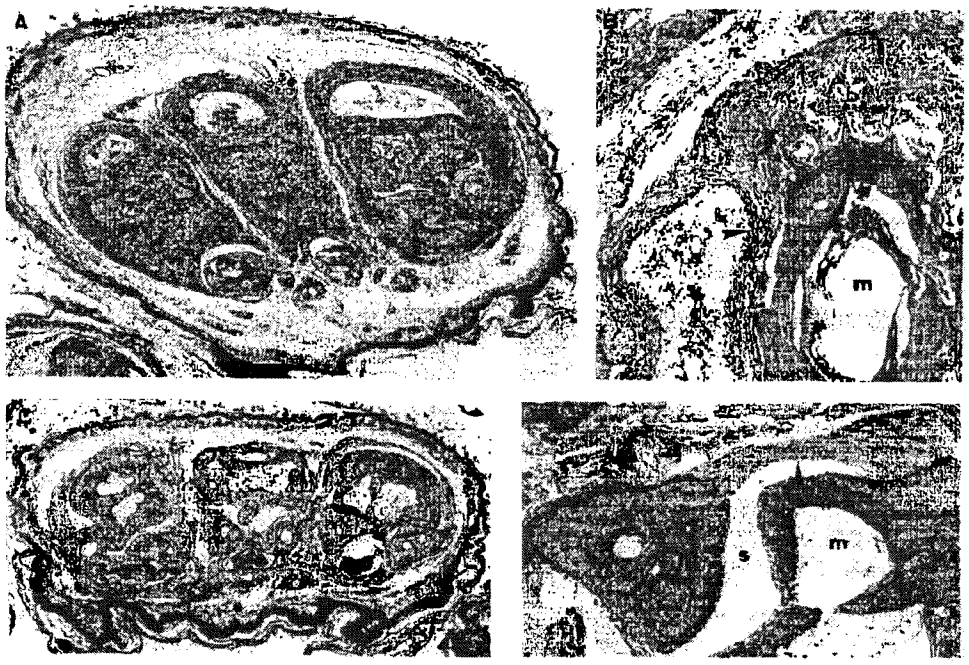

Figure 2. Histological aspect of arthritis in the hind footpad 3 months after immunization. Mice were immunized twice with collagen and treated 2 and 3 weeks after the first immunization with non-immune (A,B) or anti-TNF (C,D) IgG. At low magnification (A,C), the footpad of (A), but not (C), is enlarged by new bone formation and dermal oedema (H&E, original magnification × 30). At higher magnification, infiltration of the synovial membrane (arrow) and cavity and new bone formation are evident in (B) and absent in (D). m, medullary cavity; b, newly formed bone; s, synovial cavity (H&E, original magnification × 100).

Table 2. Anti-collagen antibodies

Treatment	Antibody titres	
	1 month	3 months
Normal IgG	346±61	16±4
Anti-TNF IgG	378±57	14±6

Mice were immunized twice with collagen and treated 2 and 3 weeks after the first immunization with anti-TNF non-immune rabbit IgG. Results are the mean ± SD of the reciprocal dilution leading to a positive optical density, defined as 2 SD above the mean value of non-immune DBA1 sera.

DISCUSSION

The present results indicate that TNF plays a critical role at a relatively early phase of collagen arthritis since the long-term evolution of the disease can be arrested by TNF antagonists given 2–3 weeks after immunization with collagen.

Similar results were obtained with two different antagonists, namely rabbit anti-mouse TNF IgG, or the human rsTNFR-β. Rabbit anti-TNF IgG were administered in bolus injection, since they are known to remain detectable in the recipient's serum up to 7 days after a single 1-mg injection and have been found to be effective in several different models of disease.[22,23] Another TNF antagonist, the rsTNFR-β has recently been found to protect mice from LPS-induced lethality.[20] Because of its small size, rsTNFR-β has a short life, and it was therefore delivered by an osmotic minipump, providing a low but permanent presence of the antagonist (Table 1). The efficacy of rsTNFR-β is of peculiar interest because this protein is of high specificity for TNF and also because, being of human origin, it provides a basis for clinical trials.

The evaluation of TNF production within the joints is difficult because of their small size. Our investigation was performed on the whole footpad, i.e. including bone, joints, striated muscle and epidermis. Since TNF mRNA is present within the normal epidermis,[23] it is likely that the TNF mRNA detected in the normal footpads proceed mainly from the epidermis, and not the joints. In the footpads of arthritic mice, TNF mRNA was moderately elevated 4 weeks after immunization in some but not all mice tested. This heterogeneity may be related to variations in the response of individual mice to collagen immunization or even to variations in the response of different joints from the same mice. This low level of TNF mRNA is in contrast to other immunopathological reactions in the skin or the lung, where a marked increase of the TNF mRNA level can be detected on Northern blots.[22,23] The present study suggests a moderate and transient increase of the TNF mRNA level in the early phase of arthritis, but none later on, when the arthritis is still active (see Figs 1 and 2). In accordance with this pattern, anti-TNF antibody had a clear-cut effect only when given at the beginning of the disease, but none later, i.e. it was preventive, but not curative.

In vivo, anti-TNF IgG does not interfere with the proliferation of T lymphocytes, the generation of cytolytic T lymphocytes, or the production of antibodies (reviewed in ref. 4). Consistent with former investigations, the anti-TNF antibody did not decrease the production of anti-collagen antibodies (Table 2). Incidence of collagen arthritis is in most cases correlated with the level of anti-collagen antibodies.[24] Mice treated with anti-TNF antibodies correspond therefore to a relatively rare instance of dissociation between the arthritis and the humoral response. The role of these antibodies in the evolution of arthritis is still controversial,[24-26] but the data in this study argue against the necessity of anti-collagen antibodies in the expression of arthritis.

In conclusion, this study demonstrates that TNF antagonists, when given at an early phase, can prevent the long-term evolution of collagen arthritis.

ACKNOWLEDGMENTS

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United States Patent [19]

Dorin et al.

[11] **Patent Number: 4,894,439**

[45] **Date of Patent: * Jan. 16, 1990**

[54] **N-TERMINAL DERIVATIVES OF TUMOR NECROSIS FACTOR PURIFIED BY MICROPOROUS PTFE MEMBRANES**

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[73] Assignee: **Cetus Corporation**, Emeryville, Calif.

[*] Notice: The portion of the term of this patent subsequent to Jun. 30, 2004 has been disclaimed.

[21] Appl. No.: **866,213**

[22] Filed: **May 22, 1986**

[51] **Int. Cl.**[4] **C07K 13/00**

[52] **U.S. Cl.** **530/351**; 530/412; 530/416; 530/417; 530/820; 530/825; 435/68; 435/70

[58] **Field of Search** 530/351, 820, 825; 435/68, 70

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Primary Examiner—Garnette D. Draper
Attorney, Agent, or Firm—Gregory J. Giotta; Elliott L. Fineman; Albert P. Halluin

[57] **ABSTRACT**

A process is disclosed for the purification of recombinantly produced biologically active proteins in which a solution containing a mixture of materials, including the biologically active protein, is passed through a continuous porous hydrophobic membrane, and the fraction enriched in the biologically active protein is recovered. Hydrophobic proteins such as TNF and recombinant ricin toxin A chain may be purified according to the process. Conditions for enhanced recovery of purified TNF using the process are disclosed. A highly purified TNF comprising 95% or greater TNF as determined by SDS-PAGE analysis, with an endotoxin content of less than 0.1 ng/mg TNF which is substantially free of pyrogens by the USP rabbit pyrogen test at a dosage range of 1.0 to 2.4×10^5 U/kg, is obtained.

9 Claims, 5 Drawing Sheets

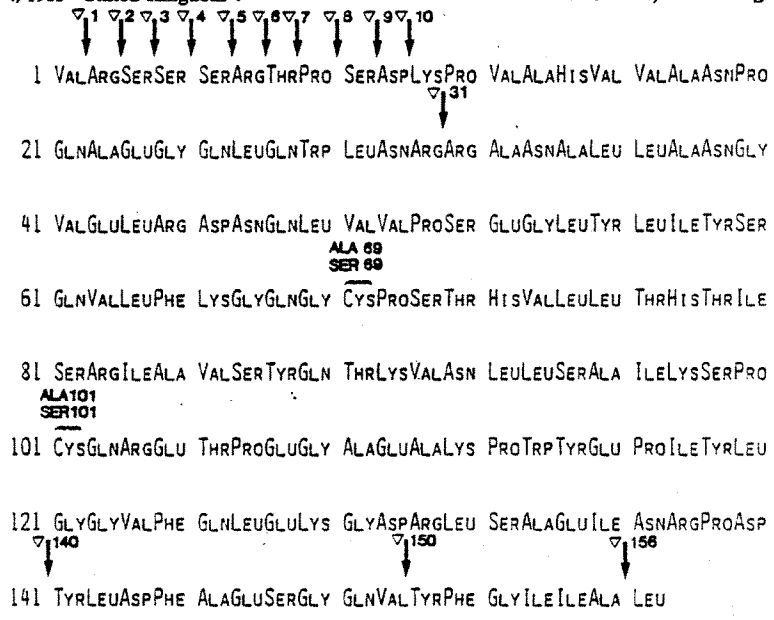

Figure 1

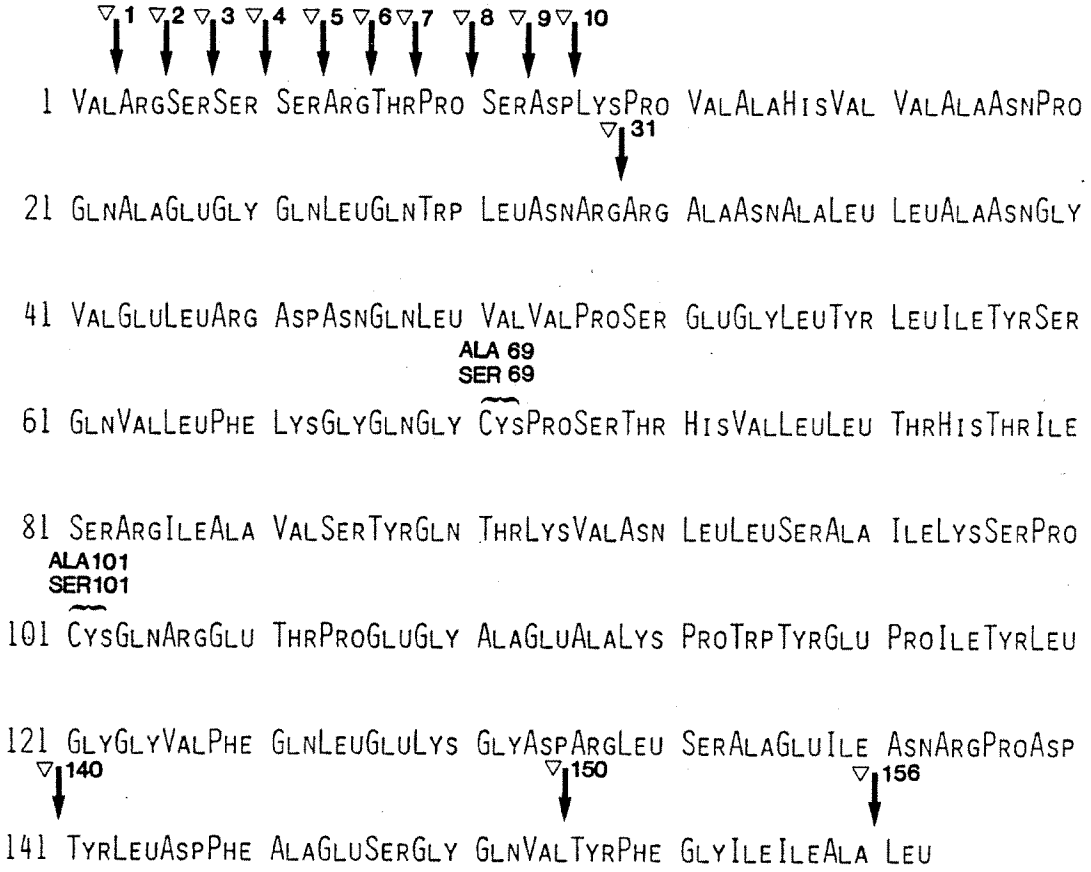
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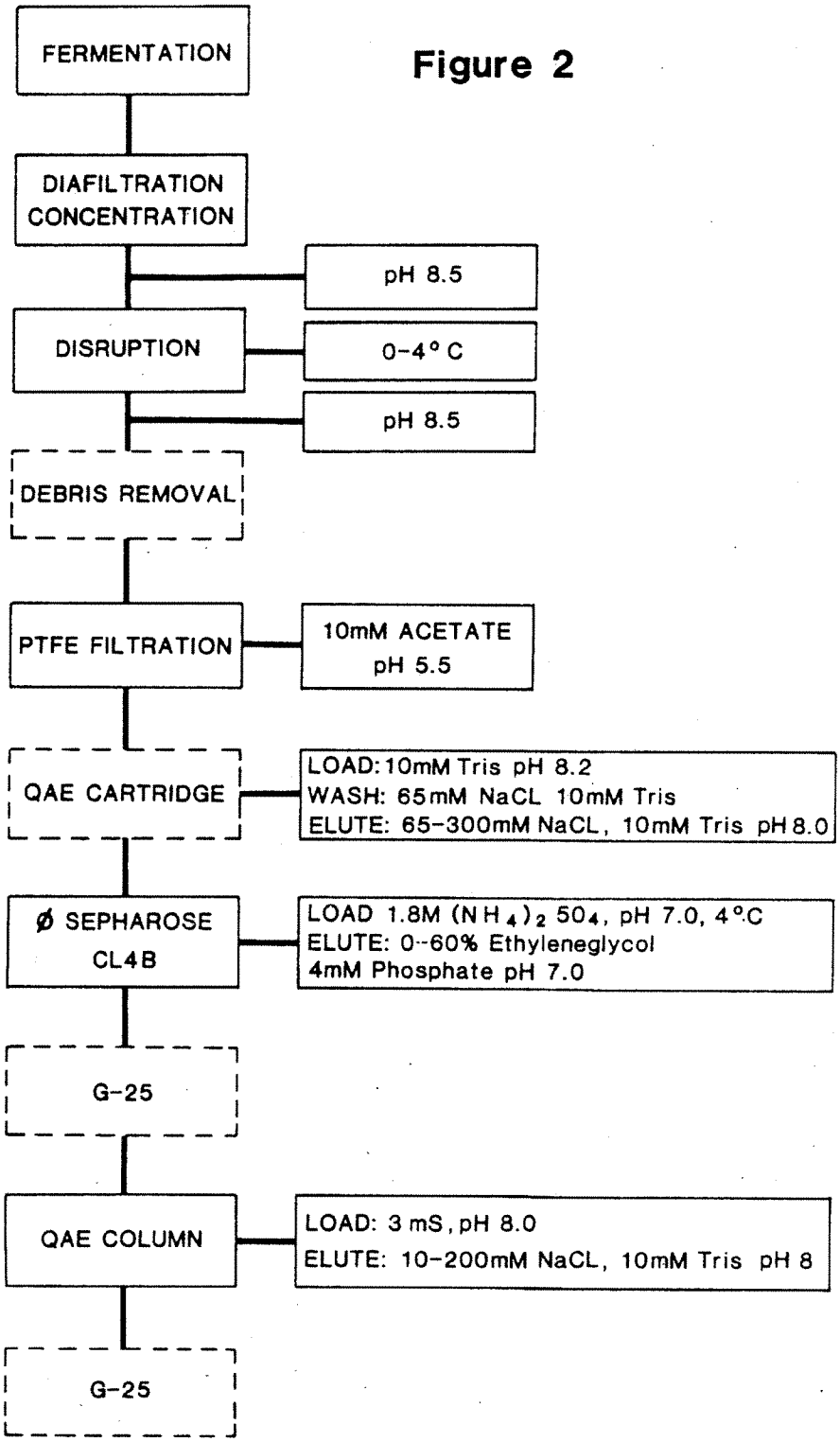
Figure 2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DISRUPTION
0-4° C
pH 8.5
DEBRIS REMOVAL
PTFE FILTRATION
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QAE CARTRIDGE
LOAD: 10mM Tris pH 8.2
WASH: 65mM NaCL 10mM Tris
ELUTE: 65-300mM NaCL, 10mM Tris pH 8.0
Ø SEPHAROSE CL4B
LOAD 1.8M $(NH_4)_2SO_4$, pH 7.0, 4°C
ELUTE: 0-60% Ethyleneglycol
4mM Phosphate pH 7.0
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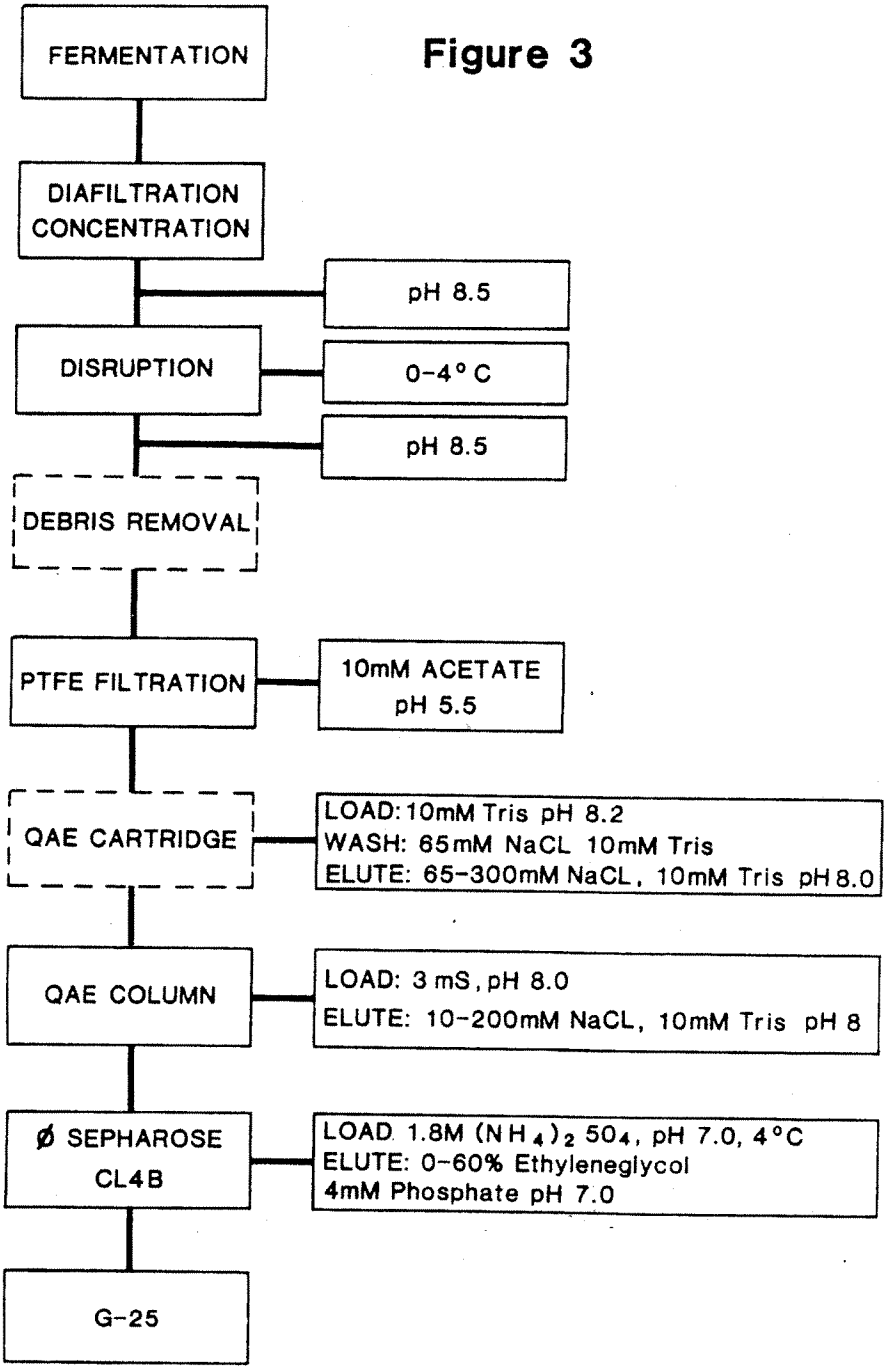
QAE COLUMN
LOAD: 3 mS, pH 8.0
ELUTE: 10-200mM NaCL, 10mM Tris pH 8
G-25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DIAFILTRATION CONCENTRATION
pH 8.5
DISRUPTION
0-4° C
pH 8.5
DEBRIS REMOVAL
PTFE FILTRATION
10mM ACETATE pH 5.5
QAE CARTRIDGE
LOAD: 10mM Tris pH 8.2
WASH: 65mM NaCL 10mM Tris
ELUTE: 65-300mM NaCL, 10mM Tris pH 8.0
QAE COLUMN
LOAD: 3 mS, pH 8.0
ELUTE: 10-200mM NaCL, 10mM Tris pH 8
Ø SEPHAROSE CL4B
LOAD 1.8M $(NH_4)_2SO_4$, pH 7.0, 4°C
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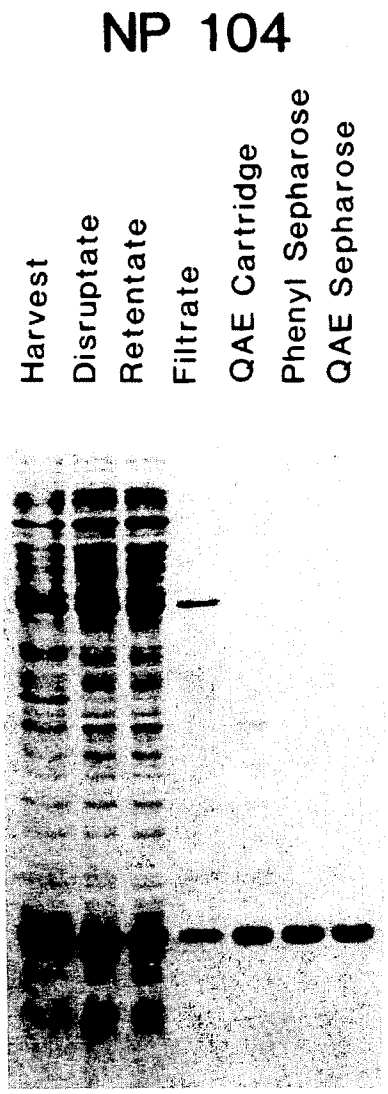
G-25

Figure 3

Figure 4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Harvest
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Retentate
Filtrate
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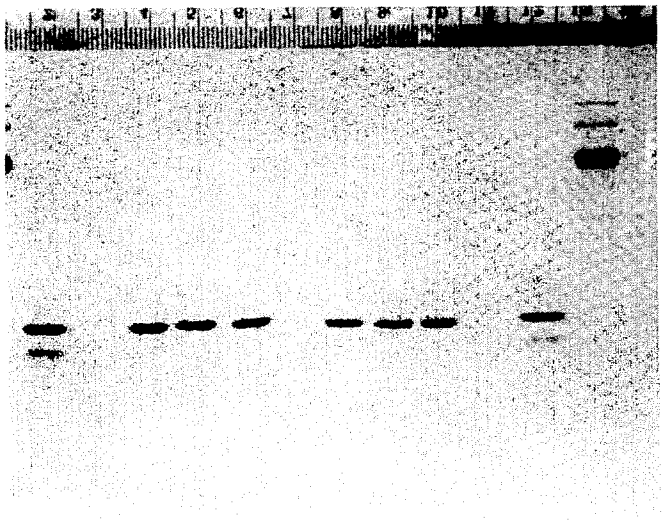
QAE Sepharose

Figure 5



LANE NO.	SAMPLE
1	MW Standard
2	TNF Control
3	Blank
4	Purified Product (reducing gel)
5	Purified Product (reducing gel)
6	Purified Product (reducing gel)
7	Blank
8	Purified Product (nonreducing gel)
9	Purified Product (nonreducing gel)
10	Purified Product (nonreducing gel)
11	Blank
12	TNF Control
13	MW Standard
14	Blank

N-TERMINAL DERIVATIVES OF TUMOR NECROSIS FACTOR PURIFIED BY MICROPOROUS PTFE MEMBRANES

FIELD OF THE INVENTION

This invention relates to a process for the purification of biologically active proteins. In particular, it relates to a process for the purification and recovery of polypeptides having the biological activity of tumor necrosis factor (TNF). The process disclosed herein is especially useful in the recovery of substantially pure homogeneous biologically active recombinant TNF which is free of pyrogenic activity other than that which may be caused by TNF itself. The invention also relates to the substantially pure homogeneous biologically active TNF which is free of pyrogenic activity other than that which ray be caused by TNF itself. The invention furthermore relates to a process for the purification of biologically active proteins that are hydrophobic at physiological pH, such proteins being in this regard like TNF.

BACKGROUND OF THE INVENTION

Processes for the purification of proteins are generally known and include such techniques as ion exchange chromatography, adsorption chromatography, gel electrophoresis, ammonium sulfate precipitation, and gel filtration.

Although each of these techniques is known, it is impossible to predict the extent to which any of the above-listed techniques is applicable to the purification of a given protein. Various factors including the extent of purification desired, the extent of acceptable loss of biological activity of the protein, and degree of homogeneity of the protein desired, require extensive experimentation to optimize the purification of the products.

Human TNF has been purified as a native protein from culture supernatants of induced HL-60 cells by a combination of anion exchange chromatography and reverse phase high pressure liquid chromatography (HPLC), with elution in a linear gradient of acetonitrile (Wang, A. M., et al., *Science* (1985) 228:149–154). Similar procedures had been previously employed (Matthews, N., *Br. J. Cancer* (1981) 44:418) without resulting in a homogeneous preparation. However, this technique is not optimally efficient even for the native TNF secreted from, for example, HL-60 or other TNF secreting cell lines, and is inappropriate for recombinantly produced TNF, due to substantial inactivation of TNF biological activity at low pH.

Copending U.S. application Ser. No. 792,815 filed Oct. 20, 1985 now U.S. Pat. No. 4,677,197 and assigned to the same assignee of the present application improves upon the process of Wang et al. supra. Whereas Wang et al. obtain a product that is not homogeneous by the steps of anion exchange chromatography, followed by HPLC and elution in a linear acetonitrile gradient, U.S. application Ser. No. 792,815 achieves an active homogeneous recombinantly produced TNF product. In the improved process according to U.S. application Ser. No. 792,815 a hydrophobic support is substituted for the reverse phase HPLC of Wang, M. et al., supra and Matthews, N. et al., supra.

European Patent Publication No. 168,214 published Jan. 15, 1986 discloses a process for purifying TNF by the steps of obtaining a TNF solution from cell culture supernatants or lysates, removing solids, adsorbing TNF from the remaining supernatant onto a silicate support, eluting TNF from the silicate support, chromatographing TNF on a tertiary amino anion exchange resin, and chromatographing TNF on an anion exchange resin containing quaternary ammonium substituents. Optional purification steps including chromatofocusing to concentrate and purify the product or passage through a sieving gel such as Sephadex G-25 are disclosed. As a hydrophobic support, EP Publication 168,214 discloses the use of silicate, polyolefin and alkyl Sepharose. The TNF is eluted from the silicate using a polyol, preferably ethylene glycol in a 10–30% range, with a 20% (v/v) concentration preferred. Further purification, according to the process, requires adsorption onto a tertiary or quaternary amino anion exchange resin such as DEAE cellulose, QAE Sephadex or the product sold under the tradename Mono Q. Purification to homogeneity, according to the process, is accomplished only upon further separation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or C4 reverse phase high performance liquid chromatography (HPLC). These latter steps are accompanied, however, by substantial loss of biological activity.

The present invention provides a method for purifying TNF that produces a substantially homogeneous TNF without recourse to reverse phase HPLC or SDS-PAGE electrophoresis. The method disclosed is applicable to large scale purification of TNF. When the host cell is a Gram-negative microorganism such as *E. coli* and the TNF is thus recombinantly produced, a number of host cell proteins and other substances are produced with the TNF. Such co-produced materials include endotoxins and pyrogenic materials that must be selectively removed from the TNF. The use of a filtration step through a hydrophobic porous matrix, offers substantial recovery of active TNF proteins and substantially complete removal of host cell proteins, endotoxins and pyrogens when the host cell is a Gram-negative microorganism, before the use of any chroaatographic techniques in purifying the TNF. As a result, large scale recovery of the material is possible.

BRIEF DESCRIPTION OF THE INVENTION

The process according to the invention is used to obtain a partially purified TNF from a TNF containing fluid obtained from a recombinant host and comprises the step of passing the TNF containing fluid through a continuous hydrophobic porous matrix, and recovering the partially purified TNF therefrom.

In another aspect, the invention includes the partially purified TNF produced by the process. The partially purified TNF comprises a least about 20% of the TNF produced by the recombinant host cells, and about 40 to 50% of the total recovered protein, and has an endotoxin content of 10 ng/ml-10 μg/ml.

In another aspect, the invention is a process for obtaining a purified TNF from a TNF containing fluid obtained from a recombinant host cell comprising the steps of passing the TNF-containing fluid through a continuous hydrophobic porous matrix to produce a partially purified TNF, further purifying said partially purified TNF by at least one hydrophobic interaction matrix chromatography step and at least one anion exchange matrix chromatography step, and recovering a purified TNF having a TNF content of at least 95% as

determined by SDS-PAGE analysis and an endotoxin content of less than 0.1 ng/mg TNF.

In one embodiment of this process, the anion exchange chromatography step preceeds the hydrophobic interaction matrix chromatography step.

In another embodiment of this process, the hydrophobic interaction matrix chromatography step preceeds the anion exchange chromatographic step.

Other optional steps of the process according to the invention include size exclusion chromatography and concentration steps. Additional anion exchange chromatography steps may be used in the further purification as is disclosed in greater detail hereinbelow.

In yet another aspect, the invention is a purified recombinant TNF composition having a TNF content of at least 95% as determined by SDS-PAGE analysis, an endotoxin content of less that about 0.1 nanograms/mg, said TNF being substantially free or pyrogens as determined by the USP rabbit pyrogen test at a dosage range of 1.0 to 2.4×10^5 U/Kg. The TNF produced is substantially similar to mature TNF or may contain modifications to the molecule, particularly N- terminal sequence deletions and substitution in amino acids.

In another aspect, the invention relates to a process for obtaining a partially purified TNF under pH conditions that reduce hydrolysis of the TNF. The pH is controlled so that it is greater than 5.5 during the first stage of the process in which TNF-producing cells are disrupted, the cell debris is removed therefrom, and the retaining fluid is diafiltered through a hydrophobic porous matrix, preferably a continuous hydrophobic porous matrix, to produce a filtrate.

In still another aspect, the invention relates to a process for obtaining partially purified biologically active proteins wherein such proteins are produced in a recombinant host, comprising the step of passing a fluid containing the biologically active protein through a continuous hydrophobic porous matrix and recovering the partially purified biologically active protein.

In yet still another aspect, the invention relates to a process for obtaining partially purified biologically active proteins that are hydrophobic at physiological pH wherein such proteins are produced in a recombinant host, comprising the step of passing a fluid containing the biologically active protein obtained from a recombinant host through a continuous hydrophobic porous matrix and recovering the partially purified biologically active protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the amino acid sequence of mature TNF and a number of N-terminally deleted muteins.

FIG. 2 is a flow diagram of a preferred TNF purification process. Optional steps in the preferred process are denoted by a broken line.

FIG. 3 is a flow diagram of an alternate preferred TNF purification process.

FIG. 4 is an SDS-PAGE gel of the purified product after each step of the purification process as shown in FIG. 2.

FIG. 5 is a silver stained SDS-PAGE gel of the final production run TNF.

DETAILED DESCRIPTION OF THE INVENTION

General Terms and Techniques

As used herein, the term "tumor necrosis factor" refers to a molecule that is substantially equivalent to the amino acid sequence of FIG. 1 and is capable of selective cytotoxicity against tumor cells. Such selective cytotoxicity according to the definition herein, is demonstrated by activity in the in vitro cytotoxicity assay based on the continuous murine connective tissue cell line L-929 as described in PCT Publication WO 86/02381, published Apr. 24, 1986 assigned to the same assignee of the present invention and incorporated herein by reference.

The amino acid sequence of TNF is shown in FIG. 1. The sequence of FIG. 1 represents the mature or native form of human TNF. A "substantially equivalent" amino acid sequence of TNF means the amino acid sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause an adverse functional dissimilarity between the altered or mutein form of the protein and native form. "Adverse functional dissimilarity" is manifest by an altered form of TNF if, in purified form, its activity in the L-929 in vitro cytotoxicity assay is destroyed. Further, individual amino acid residues in the protein may be modified by oxidation, reduction, or other derivatization, or the protein may be cleaved to obtain fragments that retain activity. Such alterations that do not destroy activity in the L-929 in vitro cytotoxicity assay mentioned above do not remove the protein sequence from the definition of TNF.

Within the foregoing definition of TNF are analogs of TNF specifically or randomly altered wherein the altered forms exhibit the selective cytotoxicity mentioned hereinabove. Such active TNF analogs or muteins ray exhibit improved properties such as increased potency in cytotoxicity assays, greater homogeneity when produced by a recombinant host, or improved processing characteristics in purification. Such TNF analogs ray also have additional functionalities not obtained in the native form, e.g., conversion of a cysteine residue leaving a free sulfhydryl in the unaltered cysteine residue that may be used to couple TNF to other moieties by formation of a disulfide or thioether bond.

Forms of TNF that are inactive in the in vitro cytotoxicity assay mentioned above may also be formed by random, site-specific or deletion mutagenesis. Although such forms do not fall within the definition of TNF herein, such forms may be useful for purposes other than causing cytotoxicity to tumor cells or direct therapy of patients having tumors susceptible to cytotoxic properties of TNF. Such forms of TNF may still potentiate or synergize the activity of other active lymphokines, for example, interleukin-2 and gamma interferon. Forms of TNF inactive in the in vitro cytotoxicity assay mentioned above may nevertheless be purified by the process disclosed herein.

Specific examples of TNF analogs include N-terminally deleted species of the protein including those having deletions of the N-terminal **1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14,** and **31** amino acids as shown in FIG. 1. Also included are species of TNF in which any or all of the cysteine residues of the TNF molecule have been converted to serine or other neutral amino acids, for example, glycine or alanine. U.S. patent application Ser. No. 698,939 filed Feb. 7, 1985 assigned to the assignee of the present patent, incorporated herein by reference, discloses cysteine depleted muteins of TNF.

Deletions of C-terminal residues of TNF have also been disclosed and the mode for carrying out such deletions of up to 17 C-terminal amino acids of TNF is found in U.S. patent application Ser. No. 760,661 filed

July 30, 1985, now U.S. Pat. No. 4,677,063 which is assigned to the assignee of the present invention and is incorporated herein by reference.

As to notation, for convenience, the protein having the amino acid sequence numbered 1–157 in FIG. 1 will be used as a reference and designated herein mTNF (mature TNF). All other amino acid sequences having homology with mTNF and showing TNF biological activity are referred to as "muteins" of mTNF and are denoted as to their differences from mTNF using the numbering of residues shown in the figure. For example, muteins which have substitutions for cysteine at position 69 will be denoted using the substituted residue and the position number, e.g., peptides having a serine in place of the cysteine at position 69 are designated ser_{69} TNF. If a residue is simply missing, it is renamed as a des-residue so that, for example, the mutein wherein the serines at positions 3 and 4 are deleted is designated des-ser_3des-ser_4TNF. Muteins which lack segments of amino acids at the N- or C-terminus are denoted according to the terminus affected. N-terminus deletions lacking a number of amino acids are denoted followed by ∇, the number of amino acids missing. For example, muteins which lack one N-terminal amino acid as compared to the protein shown in FIG. 1 are designated ∇1TNF. For deletions at the C-terminus, the ∇ will be followed by the number of the last remaining residue and a minus sign. Thus, for the mutein having 7 amino acids removed from the C-terminus, the designation would be ∇ 150-TNF. Where combinations of the foregoing alterations are made, the designation shows all of them, e.g., ∇ 1des-ser_3des-ser_4ser_{69} ∇150-TNF.

Not all muteins of TNF are recombinantly or deliberately produced. Indeed, the sequence obtained for the 22 N-terminal amino acids of the HL-60 secreted TNF contains minor modifications in the primary structure, although both native and recombinant proteins exhibit TNF activity. Specifically, the recombinant sequence has an additional pair of serine residues preceding the serine at position 5 before resuming the homology between positions 4–12 of the HL-60 derived protein and positions 6–14 of the deduced sequence.

As used herein, the term "TNF" is intended to include multimeric forms. TNF is known to form aggregates or multimers, predominantly dimers. Such multimers are selectively cytotoxic and are suitable for in vivo use. The TNF produced by the process according to the invention is substantially a homogeneous composition of monomeric TNF on SDS-PAGE electrophoresis.

As used herein, "chromatography" means that a subject mixture is treated with an adsorbent or other support matrix and then eluted, usually with a gradient or other sequential eluant, as opposed to a simple one step process. Material eluted from the support matrix is designated eluate. The sequential elution is most commonly done by placing the support matrix in a column and supplying an eluting solution which changes its character either stepwise or preferably by gradient. However, other methods may be used, such as placing the support matrix in a filter and sequentially administering eluants of differing character.

As used herein, "by chromatography" means that this elution must be done so as to elute less than all of the materials retained by the support matrix at one time. Thus, if done batchwise, the elution must be accomplished so as to selectively remove only the desired component while leaving the remaining materials retained by the support matrix or must comprise more than one step in which, for example, the desired component is selectively removed by first eluting the undesired component, followed by eluting the desired component retained by the support matrix.

The term "continuous hydrophobic matrix" as used herein is intended to encompass hydrophobic membranes. Such membranes are exemplified by polymers of lower alkylenes and substituted alkylenes. Polypropylene and polyethylene are examples o-f the former. Polytetrafluoroethylene is an example of the latter.

By "porous" is meant that the membrane has openings therein that substantially traverse the membrane. In short, a porous membrane has holes in it that go through the membrane. Such porous membranes can filter materials, allowing some portion of the material to pass through the membranes and retaining the remaining portion. "Porous" as used herein is not intended to mean pitted, i.e., having openings that do not go through the membrane.

As used herein "hydrophobic interaction matrix" means an adsorbant that is a hydrophobic solid such as polystyrene resin beads, rubber, silicon coated silica gel, or cross linked agarose sufficiently substituted with hydrophobic functional groups to render the material hydrophobic. Alkyl substituted agarose and aryl substituted agarose such as phenyl or octyl agarose are examples. Such alkyl and aryl substituents are referred to herein as hydrocarbyl. Materials to be chromatographically separated on a hydrophobic interaction chromatography (HIC) matrix are first sorbed to the HIC matrix in a high salt solution and are desorbed from the HIC matrix by elution in a low salt concentration solution or a hydrophobic solvent such as a polyol.

As used herein "anion exchange matrix" means a solid or gel support matrix that is charged in aqueous solutions. The support matrix may be agarose sufficiently substituted with amine functional groups to have a net charge in aqueous solutions. The material to be sorbed is bound to the anion exchange matrix in a low salt solution and is eluted from the anion exchange matrix in a high salt eluant containing anions such as chloride ion which bind to the anion exchange matrix and displace the sorbed material.

As used herein, "mixture" as it relates to mixtures containing TNF, refers to a collection of materials which includes TNF, but which also include other proteins. If the TNF is derived from recombinant host cells, the other proteins will ordinarily be those associated with the host. Where the host is bacterial, the contaminating proteins will, of course, be bacterial proteins. Furthermore, if the bacterial host is Gram-negative, endotoxins or lipopolysaccharide may be present. These endotoxins are routinely removed in the purification process according to the invention. However, if the TNF is associated with native sources, such proteins will be mammalian. Other non-proteinaceous materials may also be present, but generally do not constitute a purification problem.

By "high salt concentration conditions" is meant an aqueous solution wherein an ionic substance is present to create conditions of high ionic strength. Ionic strength is defined, as is understood in the art, to be calculated from the putative concentrations of the various ions placed in solution modified by their activity coefficients. Workable high salt concentrations are typified by solutions containing high concentrations of ammonium sulfate. However, other salts such as sodium

chloride, potassium chloride, sodium sulfate, sodium nitrate, or sodium phosphate can be used instead, provided solubility permits and provided the same ionic strength can be obtained.

As used herein, the term "host" refers to a cell producing TNF. Such host cells may be mammalian cells that produce TNF from DNA sequences coding for TNF that are endogeneous to the genome of the cell in its native state. Preferably, the host cell will be a recombinant host cell, i.e., one into which a TNF-encoding DNA sequence has been introduced by means of recombinant molecular biological methods. Such a host cell within the definition includes eukaryotic hosts, including, for example, such mammalian cells as mentioned above into which, in addition, a TNF-encoding DNA sequence has been introduced.

Alternatively, the host cell will be a eukaryotic microorganism such as a yeast or fungus into which the DNA sequence encoding TNF has been introduced. Most preferred are prokaryotic host cells, such as members of the genuses Bacillus, Streptomyces, and Escherichia. Among Bacillus hosts, *Bacillus subtilis* is preferred. Within the genus Escherichia, *E. coli* is preferred.

The term "diafiltration" and "diafilter," as used herein, refers to a filtration process wherein the material to be filtered is maintained in a volume of liquid. Solid retained by the filter is designated retentate; liquid material passing through the filter is designated filtrate. In diafiltration processes, as the liquid filtrate is removed from the retentate across the filtering medium, liquid volume is replaced on the retentate side of the filter, preferably at a rate equal to the rate filtrate is removed. As a result, material that is capable of passing through the filter is washed from the retentate.

As used herein the term "biologically active proteins that are hydrophobic at physiological pH" refers to proteins that are hydrophobic, but soluble in a pH range between about 7.2 and 7.6. Such biologically active proteins are typified by recombinant TNF and recombinantly produced ricin toxin A chain and have the characteristic of binding to hydrophobic supports such as phenyl-TSK and phenyl agarose. The production of recombinantly produced soluble ricin toxin A chain is described in U.S. patent application Ser. No. 837,583 filed Mar. 7, 1986 and assigned to the assignee of the present patent application, and is herein incorporated by reference.

GENERAL METHOD AND PREFERRED EMBODIMENT

The process for purifying TNF according to the invention comprises two stages as shown in the flow diagram of FIG. 2. In the first stage of the process according to the invention, TNF is partially purified from a fluid containing TNF and other cell products. This TNF-containing fluid, obtained from collected disrupted TNF-producing host cells by removing the cell debris of the disrupted host cells, is filtered through a substantially continuous hydrophobic porous matrix to produce a filtrate containing a mixture enriched in TNF. To enhance the yield of the TNF in the process according to the invention, the pH conditions of the collected TNF-producing cells, their disruption and the removal of cell debris are controlled to reduce hydrolysis of the TNF. The pH conditions of the filtration of the TNF-containing fluid may be similarly controlled to maximize the TNF yield.

In a preferred embodiment of the process for purifying TNF according to the invention TNF-producing host cells are collected in a volume of fluid and the pH of the collected host cells is adjusted or maintained at a pH that reduces hydrolysis of TNF. In all of the subsequent steps of the first stage the pH of the material produced from the host cell is similarly adjusted. The collected host cells are disrupted and disrupted cell debris is removed leaving a fluid containing TNF and other cell products. This TNF-containing fluid is then passed through a continuous porous hydrophobic matrix to produce a filtrate containing a mixture enriched in TNF or a partially purified TNF.

In the second stage of the process, the mixture enriched in TNF is subjected to at least two chromatographic steps, one on a hydrophobic interaction (HIC) matrix and one on an anion exchange matrix. In one embodiment of the process, illustrated in FIG. 2, the mixture is first chromatographed on an HIC matrix and selectively eluted therefrom. The eluate so produced is substantially free of residual proteins and nucleic acid degradation products such as nucleotides and nucleosides. The material selectively eluted from the HIC matrix is then further chromatographically purified on an anion exchange matrix. Optionally, the material selectively eluted from the HIC matrix is desalted prior to this anion exchange chromatography step. A fraction high in TNF is selectively eluted from the anion exchange column using an appropriate salt. Optionally, depending upon the type and amount of salt solution used to elute the fraction high in TNF from the anion exchange matrix after the chromatography on the HIC matrix, the fraction high in TNF may require desalting on an appropriate material. In an additional option, illustrated in FIG. 3, prior to chromatographing the mixture on the HIC matrix, the mixture is chromatographed on an anion exchange matrix, and eluted therefrom with an appropriate salt solution.

The filtrate containing a mixture of TNF or partially purified TNF produced by the first stage of the process represents at least about 20% of the theoretical yield of TNF produced by the host cells. More typically the partially purified TNF amounts to between 40 and 50% of the theoretical yield of TNF produced by the cells. The mixture comprises from 40% to 70% TNF as a fraction of total protein recovered. The endotoxin level in the mixture contains between about 10 ng/ml and 10 μg/ml of the mixture.

The purified TNF recovered at the end of stage 2 of the process has a TNF content of at least 95% as determined by SDS-PAGE analysis and an endotoxin level of less than 0.1 ng/mg TNF. TNF level frequently exceeds 98% and may exceed 99%. In addition, the material is substantially free of pyrogens as determined by the USP rabbit pyrogenicity test at a dosage in a range between 1×10^5 and about 2.4×10^5 U/Kg.

In the process according to the invention, the host cell may be any one of a TNF-producing mamamalian cell, a recombinant TNF-producing eukaryotic cell. including a recombinant mammalian cell, a recombinant eukaryotic microorganism such as a yeast, for example, Saccharomyces, or other fungus such as those of the genus Aspergillus, a recombinant prokaryotic microorganism such as the Gram-positive microorganism of the genus Bacillus, including *B. subtilis* or *B. cereus,* or genus Streptomyces or Gram-negative microorganism such as the genus *Escherichia, Serratia* and the like. *E. coli* is particularly preferred.

The host cells, preferably *E. coli* transformed with a plasmid having a DNA sequence encoding TNF which is expressed by the host cell to produce TNF, are grown in a suitable growth medium to a desired cell density typically measured by optical density (OD) at 680 nm. Optical densities between about 20 and 40 are typical and an OD of about 30 is preferred.

For expression of TNF in *E. coli* transformed with a plasmid expression vector that expresses TNF under the control of most bacterial promoters, *E. coli* strain MM294, (Talmadge, K., et al., *Gene* (1980) 12:235 and Messelson, M., et al., *Nature* (1968) 217:1110), is used as the host. An appropriate MM294 strain has been deposited in the American Type Culture Collection under accession number 39,894 on Oct. 19, 1984. In such expression vectors under control of the tryptophan (trp) promoter, the trp concentration in the medium is carefully controlled to become limiting at the desired host cell density at the time TNF expression is desired.

For expression under the control of the P_L promoter and gene N ribosome binding site, *E. coli* strain K12 MC1000 lambda lysogen $N_7N_5CI857SusP_{80}$, ATCC accession number 39,531 is used. Expression of TNF under control of the P_L promoter is obtained by shifting the temperature from 30° to 42° C. when the desired OD of the culture is obtained.

The composition of the growth medium will, of course, depend upon the particular microorganism used. In general, an aqueous medium is used that contains assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and such amino acids and purine or pyrimidine bases as required.

After the cells are harvested from the culture medium, they may be concentrated, if necessary, by filtration, centrifugation or other means. If TNF is produced as an intracellular product, it is preferred to remove from the harvested cells substantially all of the culture medium by washing the cells in an appropriate solution adjusted for pH and osmolarity so as not to prematurely break the cells or hydrolyse the desired protein product. Various means may be used to wash the cells, including low speed centrifugation so as not to shear the cells, alternating with cell washing or diafiltration. Diafiltration is preferred.

The pH of the concentrated cell suspension is also adjusted in a range that reduces hydrolysis of the TNF to be purified. pH adjustment is carried out by adding sufficient amounts of acid, preferably HCl, or base, preferably NaOH, at a concentration sufficient to reach the desired pH without degrading the protein or cells. A pH range between 5.5 and 9.5 is maintained. For TNF purification, alkaline pH is preferred and a pH of 8.5 is most preferred.

The cell membranes of the collected host cells are disrupted to release intracellular TNF. The choice of cell disruption methods will depend largely on the amount of cells harvested, but any conventional technique will be sufficient. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling ray be used. Preferred methods are sonication or homogenization with a cell homogenizer such as a Manton-Gaulin homogenizer. Cell disruption should break substantially all of the cells so that effectively no intact cells retain in the processing of the material during subsequent steps. The end point of the disruption step may be determined by monitoring the drop in the optical density of the disrupted cells to an optical density of between about 65% and 85% of the initial OD. The pH of the disrupted cell, or disruptate, will also be monitored and adjusted as necessary. pHs between 5.5 and 9.5 are acceptable. Alkaline pHs within the range are preferred and a PH of 8.5 is most preferred. pH adjustment of the disruptate may be made with suitable buffered pH solutions.

To further reduce TNF hydrolysis, it is desirable to carry out the disruption with temperature control at a temperature between 0° and 10° C. A temperature between 0° and 4° C. is preferred. Subsequent to the disruption of the host cells, the particulate matter is separated from the liquid phase of the disruptate by any conventional separation method. The removal of particulate matter at this point in the purification process is desirable because it has been discovered that cellular components associated with the cell debris can cause hydrolysis of the TNF produced by the cell.

Various means are known for removing particulate matter from the cell disruptate. Flocculating agents, such as calcium ion supplied from dissolved calcium chloride, may be added to the disruptate to aggregate suspended particulate ratter. The aggregated particulate matter in cell debris is removed by centrifugation or settling. Various appropriate flocculating agents, such as polyelectrolytes, or caking agents, such as a product sold under the tradename Filter Aid (Whatman), may be used. Various flocculating agents will be known to those skilled in the art. Centrifugation, without the use of flocculating agents, may also be used to accumulate and separate the liquid phase from the particulate matter of the disruptate.

Alternatively, the particulate matter may be separated from the liquid phase of the cell disruptate by filtering through a nonhydrophobic filter, preferably a hydrophilic cellulose ester membrane. The pore size of the filter will be selected so as to optimize the flux of the liquid phase across the filter while retaining the particulate matter. Thus, the optical pore size will depend upon the extent to which the cell material has been disrupted and whether the cell debris have been aggregated through the use of flocculating agents. Thus, appropriate pore sizes ray range from 0.01 microns to 2 microns. Pore sizes between 0.01 and 1 microns are preferred. Prior to separation of the particulate matter from the liquid phase of the disruptate, the pH of the disruptate is once again adjusted to between pH 5.5 and 9.5. Alakline pH is preferred and a pH of 8.5 is most preferred. If filtration is the method used for separating the liquid phase from the particulate ratter of the cell disruptate, cross-flow filtration is preferred.

Following separation of the liquid phase of the disruptate from the particulate matter, the liquid phase is filtered through a substantially continuous porous hydrophobic matrix. Such hydrophobic substantially continuous matrices may be made of, for example, polymers of lower alkylenes and substituted polyalkylenes such as polypropylene and polyethylene. Polytetrafluoroethylene (PTFE) is preferred. The substantially continuous porous hydrophobic matrix will generally be in the form of a membrane having a defined pore size. Such membranes may be obtained from Dorr-Oliver, Inc., Stamford, Conn. and W. L. Gore & Associates, Inc., Newark, Del. It has been found that by varying the pH, ionic strength, membrane symmetry and membrane pore size, it is possible to elute the TNF selectively and permit a small subset of proteins, including TNF, to pass through the membrane, and thereby accumulate a fil-

trate enriched in TNF. A pH between 5.0 and 9.5 is desirable for the filtration of TNF through the hydrophobic membrane. pHs in the acidic portion of the range are preferred and a pH of 5.5 is especially desirable. In general, any buffering system may be used so long as it can maintain the pH in the desired range. Such buffers include, for example, acetate, citrate and succinate. Ten millimolar (mM) acetate buffer has been used to good effect. Pore sizes sufficiently large to pass proteins of about 100,000 molecular weight may be used. Pore sizes from 0.1 to 3.0 microns are acceptable and a 1.0μ pore size is preferred.

Various filtration geometries may be used in filtering the liquid phase of the cell disruptate through the hydrophobic membrane. For large scale purifications, it is preferred to use diafiltration.

The material obtained after the filtration of the material through the hydrophobic membrane, at the end of the first stage of the purification process, is characterized by having a large content of TNF. Based on biological activity, approximately 50% of the TNF produced by the host cell can be recovered in the filtrate from the hydrophobic membrane. Of the protein in the filtrate, 40 to 60% of the total protein has been identified as TNF. This represents a four to six-fold purification of the TNF through the first stage of the purification process. In addition, endotoxin levels, as determined by limulus amebocyte lysate tests, range from 10 nanograms to 10 micrograms per ml of filtrate.

The steps of the recovery process subsequent to the first stage of the purification process are designed to separate the TNF from *E. coli* protein to a high level of purity, preferably at least about 95%, and more preferably at least about 98%. Simultaneously, these purification processes also reduce the levels of pyrogenic substances exogenous to TNF to a level acceptable for parenteral administration to patients. Such pyrogenicity as is detectable is believed to be inherent in the TNF molecule.

The subsequent steps in the purification of TNF are chromatographic purification steps as defined hereinabove. In an optional step the filtrate of TNF and proteins obtained at the end of the first stage is concentrated. The filtrate of TNF and protein may be concentrated by chromatography on an anion exchange matrix. The mixture is adjusted to a pH appropriate for use on the anion exchange matrix in an acceptable buffer. In general, mild alkaline pH in a range between 7.5 and 8.5 is preferable, and a pH of 8.2 is most desired. Appropriate buffers include tris(hydroxymethyl)aminoethane, glycylglycine, and triethanolamine. Ten mM Tris is preferred. Alternatively the filtrate of TNF and proteins obtained at the end of the first stage may be concentrated by ultrafiltration using a filter of a pore size sufficiently small to prevent TNF from passing through the filter. A pore size sufficient to retain proteins of molecular weight above 10,000 daltons is adequate for this purpose. In addition, the filter must be made of a material to which the TNF does not significantly adsorb so that the TNF remains substantially in the retentate. A mildly hydrophobic membrane is generally acceptable. Polysulfone membranes are preferred.

In addition to concentrating the filtrate of TNF and protein, the anion exchange matrix may be selected from those that are able to selectively remove nucleic acid degradation products such as nucleotides and nucleosides by permitting the TNF from the mixture to be selectively eluted from the anion exchange matrix in a filtrate that is substantially free of nucleic acid. Among the appropriate anion exchange resins are those that contain bound tertiary and quaternary ammonium ion. Such anion exchange matrix material is typified by QAE-agarose, QAE-cellulose, and DEAE-agarose. The mixture is loaded on an anion exchange matrix. Prior to eluting the TNF from the anion exchange matrix, the matrix is washed with a buffered salt solution, for example, NaCl. The NaCl wash selectively elutes the nucleic acid degradation products from the anion exchange matrix while selectively retaining TNF on the column at the molar concentration of the salt wash. Molar anion strengths of the wash will generally be less than 65 mM.

After the column is washed, the TNF is eluted form the anion exchange matrix in an appropriate increasing gradient of anion that is applied to the column. The eluate is monitored by optical means for absorbance in the range of 280 nm to detect the fractions of eluate containing protein as they elute from the column. The protein is eluted from the column in a salt gradient that ranges between 65 at 300 mM. Linear and stepwise gradients may be used to advantage in the process. Chloride is the preferred anion, although phosphate ion and sulfate ion are acceptable.

Provided that sufficient amounts of nucleic acid degradation products and protein have been removed in the first stage of filtering the TNF-containing fluid through the hydrophobic porous matrix, it may be desirable to completely dispense with the initial optional second stage purification step using the anion exchange column. In either event, the filtrate from the hydrophobic membrane, or the eluate from the anion exchange column, is next chromatographed on a hydrophobic interaction matrix. A number of hydrophobic interaction matrices are known and include, for example, phenyl-TSK, a resin commonly used as an HPLC support column. In general, appropriate hydrophobic supports are comprised of alkyl, phenyl, or other essentially hydrocarbyl substituents of sufficient hydrocarbon content to be hydrophobic, bound to a polymer matrix, usually a carbohydrate. Other hydrophobic polymers include polyolefins. More Preferred are alkyl agaroses as the hydrophobic interaction matrix. Phenyl agarose is particularly preferred, although octyl agarose may also be employed. The most advantageous form of alkyl agarose is one in which agarose content is between about 3.5 and about 8%, and is crosslinked. More preferred are phenyl aragose resins in which the agarose content is between 4 and 6% and crosslinked. Most preferred is a phenyl agarose having 6% crosslinked agarose. A product of Pharmacia Corporation (Uppsala, Sweden), called Fast Flow Phenyl Sepharose, is particularly preferred.

In addition, macroporous substrates that are alkyl or phenyl substituted are in general of sufficient hydrophobicity to serve as a hydrophobic interaction matrix. In addition, various silicas also meet this criterion. In general, any hydrophobic material that will bind TNF under high salt conditions within the pH range of from 4 to 9 may be used, although those that bind in a pH range between 5 and 9 are preferred.

Prior to loading the column with the mixture or filtrate, the column is equilibrated with a high salt solution. Workable high salt concentrations are typified by solutions containing high concentrations of ammonium sulfate. Other salts such as sodium chloride, potassium chloride, sodium phosphate, sodium sulfate, magnesium

sulfate, and sodium nitrate, can be used provided that solubility permits, and provided that the same ionic strength can be obtained. In a preferred mode, the column is equilibrated with ammonium sulfate, in a range between 1.5 and 2 molar. Preferably, the sodium sulfate is used at a concentration of 1.8 molar. Four molar sodium chloride can also be used.

The eluate or filtrate is brought to high salt concentration as defined above and is loaded on the column. In general, the high salt concentration is achieved by adding ammonium sulfate to between 1.5 and 2 molar, preferably 1.8 molar. The pH of the high salt solution is maintained between about 5 and 7. A PH of 5.5 is preferred. The column and the material bound thereto al this high salt concentration is maintained at a temperature in a range between 0° and 25° C. It is generally preferred that the material and column be maintained at a temperature well below 25° C. during this step, with 4° C. being preferred.

The TNF protein is eluted from the hydrophobic interaction matrix at a low salt concentration. The particular salt concentration will depend upon the mutein form of the TNF molecule chromatographed and on the particular hydrophobic interaction matrix used. In general, the TNF material elutes as the salt concentration drops. The particular salt concentration at which the TNF elutes will also depend somewhat upon the buffer used.

Various materials may be used to elute the TNF from the column, including various chaotropic agents and nonionic detergents. Polyols ray also be used so long as they remain substantially flowable at the temperature at which the column is maintained. Propylene glycol and ethylene glycol may be used. A linear gradient of ethylene glycol in an appropriate buffer in a range of from 0 to 60% ethylene glycol is preferred. As indicated above, the buffer may vary and the pH may range between 5 and 8. Four mM acetate buffer may be used at a pH of about 5. A pH of 5.5 is preferred.

Optionally, the material eluted from the hydrophobic interaction matrix may be desalted. Preferably, the material is desalted using a size exclusion resin such as G-10, G-15 or G-25 Sephadex.

Once eluted from the hydrophobic interaction matrix, the protein solution or optionally desalted protein solution is chromatographed on an anion exchange matrix. Any anion exchange matrix can be used which will selectively bind the protein and allow the chaotropic agent or detergent to pass so that the bound protein may be eluted. Such anionic exchange matrices are well known to those skilled in the art and in ge co substituted amines in an agarose or cellulose matrix. Trisubstituted and quaternary substituted amines are particularly preferred. Diethylaminoethyl (DEAE) agarose is one such ionic exchange matrix. Quaternary substituted agarose and cellulose are also suitable. A convenient commercial quaternary ammonium anionic exchange medium is sold under the tradename Fast Flow Q Sepharose (Pharmacia, which is a bound quaternary ammonium ion containing matrix.

As mentioned above, prior to loading the protein solution eluted from the hydrophobic interaction matrix onto the anion exchange matrix, the protein solution ray be optionally desalted. If the Protein solution eluted from the hydrophobic interaction matrix is not desalted, then the eluate is diluted with double distilled deionized water to decrease the ionic strength of the solution so that the protein will bind to the column. Ionic strength of the material is determined by monitoring the conductivity of the solution so that it is below 3 millisiemens (mS). In general, a conductivity of approximately 2 mS is preferred. The pH of the solution is adjusted to between about 7 and 9. A pH of 8 is preferred. Prior to loading the pH-adjusted eluate on the column, the column is equilibrated with buffer. Sodium phosphate, Tris sulfate or Tris chloride are appropriate. Ten mM is preferred.

A salt gradient is used to elute the TNF protein selectively from the column. A sodium chloride or sodium sulfate gradient may be used. The salt gradient is buffered with an appropriate buffer which is generally the same as the one used to equilibrate the column. The pH of the buffer is again maintained between 7 and 9, preferably at pH 8. If sodium sulfate will be used as the eluting salt gradient, for example, Tris sulfate will be used as the buffer. The salt gradient ranges between 0 mM to 200 mM.

The protein is collected in fraction of equal aliquots as it comes off the column and is monitored for protein concentration at 280 nanometers in a spectrophotometer. Optionally, in the event that Tris buffer is used, a desalting step using a sizing column is required if the material is to be used for therapeutic purposes. G-10, 15 or 25 Sephadex are appropriate size exclusion resins.

The invention will be more clearly understood in relation to the following examples which are intended by the applicant to be merely exemplary and non-limiting.

EXAMPLE I

Growth of Recombinant TNF-Producing Host Cells

A. A fermenter was filled with distilled deionized water to operating volume and the following materials were added to the indicated final concentrations: $ZnSO_4.7H_2O$, 60 μM; $MnSO_4.H_2O$, 60 μM; $CuSO_4.5H_2O$, 2 μM; $Na_3citrate.2H_2O$, 1.5 mM; KH_2PO_4, 21.6 mM; $(NH_4)hd\ 2SO_4$, 72 mM. The medium was sterilized in the fermenter. The pH of the medium was adjusted to 6.5±0.1 with KOH. 50% glucose, KOH and antifoam were added by sterile feeds to the fermenter to achieve a 5 g/l glucose concentration. The following solutions were also added to the indicated final concentrations: 100 μM $FeSO_4.7H_2O$, 20 mg/l thiamine HCl; 3 mM $MgSO_4.7H_2O$.

B. Inoculum: A stock culture of *E. coli* K12 strain DG95λ transformed with plasmid pAW740A, the tranformant having ATCC Accession No. 53332, was thawed and grown at 30° C. to an optical density of 50-100 Klett units in flasks using double strength Luria broth with 10% NaCl, 5 mg/100 ml ampicillin and frozen in vials. One raster stock vial was grown in Luria broth as above, but without ampicillin. Culture was diluted to 10% in glycerol and dispensed into vials and frozen at −70° C. to be used as working stock.

A container of the working stock was thawed, grown in 2× Luria broth at 30° C. to approximately 1 OD_{680}, then added to the fermenter to a final cell concentration of 1 mg/liter.

C. *Growth Conditions*: Temperature was maintained at 30° C.±1° C., dissolved O_2 concentration was 40% air saturation and pH was controlled at 6.8 by automatic addition of 5 N KOH. Optical density was monitored. When the culture reached an OD_{680} of 15 units, the temperature was raised to 42° C. to induce TNF production and casamino acids were added to 2%. Cells

were harvested about four hours after casamino acid addition.

EXAMPLE II

Concentration and Diafiltration

The harvested material was concentrated approximately 5-fold by circulating it under pressure past a hollow fiber microporous (0.2 μ) polypropylene membrane. Residual medium was removed by diafiltration against 5 volumes of deionized water. The retentate was kept and the pH adjusted to 8.2.

EXAMPLE III

Cell Disruption

The concentrated cell suspension was disrupted by multiple passages through a Manton-Gaulin high pressure homogenizer at 6000 to 8000 psig. After disruption the system was washed with deionized water. The disruptate and rinse water were retained and the pH adjusted to 8.2.

EXAMPLE IV

Diafiltration on Hydrophobic Membrane at pH 5.5

The cell disruptate and rinse water were pH adjusted to 5.5 with glacial acetic acid. The pH adjusted material was diafiltered against 5 volumes of 10 mM acetate buffer using a Dorr-Oliver diafiltration device and a polytetrafluoroethylene membrane having a 1.0μ pore size; Door-Oliver part number GFI16-D10868-1. The filtrate was collected.

EXAMPLE V

Diafiltration on Hydrophobic Membrane at pH 8.5

The cell disruptate and rinse water were treated as in Example IV except that a pH of 8.5 was maintained using 10 mM Tris buffer

EXAMPLE VI

Removal of Disrupted Cell Debris:Centrifugation

The cell disruptate and rinse water of Example IV were pH adjusted using Tris and NaOH to 8.2 as necessary and the flocculating agent $CaCl_2$ was added to aggregate cell debris. The aggregate was separated from the supernatant by centrifugation at 14000 xg in a Sorvall RC-3B centrifuge. The supernatant was retained and treated as in Example IV and V.

EXAMPLE VII

Removal of Disrupted Cell Debris:Diafiltration

The cell disruptate and rinse water of Example IV were pH adjusted using Tris and NaOH to 8.2. The material was diafiltered under pressure at 15 psi against 5 volumes of distilled water using a crossflow hydrophilic cellulose ester hollow fiber cartridge (Model KF-200-10, Microgon, Laguna Hills, CA). The filtrate was retained and the pH was adjusted and treated as in Example IV or V.

EXAMPLE VIII

Concentrating the Filtrate

A. Anion Exchange Chromatography

Tris was added to the filtrate of Example IV to a concentration of 10 mM and the pH adjusted if necessary to 8.2 with glacial acetic acid or NaOH. An anion exchange column containing quaternary ammonium ion (Zeta prep-250 QAE cartridge) was equilibrated with 10 mM Tris and the filtrate was loaded onto the cartridge. The loaded material was washed with 65 mM NaCl, 10 mM Tris, pH 8.0. A low molecular weight fraction absorbing at 260 nM eluted with the 65 mM salt wash. A linear 65–300 mM NaCl gradient in 10 mM Tris, pH 8.0 maintained by an Eldex gradient controller is used to elute the TNF while monitoring the eluate for protein by absorbance at 280 nM. The protein fraction elutes from the column to yield a TNF-containing eluate having a volume approximately one-twentieth of the filtrate loading volume, and substantially free of nucleic acid degradation products absorbing at 260 nM.

B. Ultra Filtration

The TNF-containing filtrate of Example IV was concentrated 10 to 20-fold by ultrafiltration on a 10,000 molecular weight cut off polysulfone membrane (Dorr-Oliver part #16-D10864-01). The TNF remained in the retentate and the retentate was subsequently treated as in Example IX.

EXAMPLE IX

Phenyl Sepharose Chromatography of Filtrate

$(NH_4)_2SO_4$ was added to the filtrate containing TNF obtained in Example IV to a concentration of 1.8 M and the pH was measured and adjusted to 7.0. The material was filtered through a 0.45 micron filter. The filtrate was loaded onto a phenyl Sepharose CL4B column (Pharmacia, Uppsala, Sweden) after first equilibrating the column with 1.8 M $(NH_4)_2SO_4$ in sodium phosphate buffer at pH 7.0. Using an Eldex gradient controller (Eldex Laboratories, Inc., San Carlos, CA), a linear gradient of 100% 1.8 M $(NH_4)_2SO_4$, in 10 mM sodium phosphate buffer, at pH 7.0 to 100% of 60% ethylene glycol in 4 M sodium phosphate buffer pH 7.0, was used to elute the protein from the column. The eluate fractions were monitored for protein concentration at 280 nM and those fractions of TNF falling within 80% of the maximum peak height on the ascending and descending legs of the plot of protein concentration of the eluate fractions were retained and pooled.

EXAMPLE X

$(NH_4)_2SO_4$ was added to the TNF-containing eluate of Example VIII.A or the retentate of Example VIII.B to a concentration of 1.8 mM, adjusted to pH 7.0 with HCl and filtered through a 0.45 μM filter. The sample was subsequently chromatographed on phenyl Sepharose CL4B as in Example IX.

EXAMPLE X

The pooled eluate from the phenyl Sepharose column is desalted by chromatography on a G-25 Sephadex column equilibrated with 10 mM Tris buffer pH 9.2. The fast eluting fraction detected by absorbance at 280 nM is collected and subsequently treated as in Example XI, XII or XIII except that the dilution with deionized water is omitted unless necessary to achieve a conductivity of less than 2.2 ms.

EXAMPLE XI

The pooled eluate fractions obtained from the phenyl Sepharose column were diluted with deionized water to a conductivity less than 2.2 mS and the pH was adjusted to 8.2 with NaOH. A QAE Sepharose column (Pharmacia) was equilibrated with 10 mM sodium phosphate buffer at pH 8.2 prior to loading the diluted pooled fractions on the column. A linear gradient of 10 mM to 200 mM sodium phosphate buffer PH 8.2 was used to elute the TNF protein from the column. The eluate is monitored at 280 nM for protein concentration of the

eluate fractions. The TNF peak falling within 90% of the maximum height on the ascending and descending legs of a plot of eluate fraction protein concentration is retained.

EXAMPLE XII

The pooled eluate fractions from the phenyl Sepharose column were diluted as in Example XI except that 10 mM Tris at pH 8.0 was used. The QAE Sepharose column was equilibrated with 10 mM Tris.HCl buffer at pH 8.0 prior to loading the diluted pooled fractions on the column. A linear 10 to 200 mM NaCl gradient in 10 mM Tris pH 8.0 was used to elute the TNF from the column. The eluate was monitored and fractions pooled as in Example XI.

EXAMPLE XIII

The pooled fractions from the phenyl Sepharose column were handled as in Example XII except that 10 mM Tris.SO_4 pH 8.0 was used to dilute the sample and equilibrate the column. A 10 to 200 mM linear sodium sulfate gradient in 10 mM Tris.sulfate pH 8.0 was used to elute the TNF. Protein concentration was monitored and the TNF fractions were pooled as in Example XI.

EXAMPLE XIV

Pooled eluates of Examples XII and XIII are desalted using a G25 Sephadex column equilibrated with 10 mM $NaPO_4$ at pH 8.0. The fast eluting fraction was monitored for protein concentration at 280 nM , pooled and retained.

EXAMPLE XV

Determination of TNF Potency-Assay of Biological Activity

TNF activity is quantitatively measured using an in vitro cell cytoxicity assay utilizing a TNF sensitive murine L-929 fibroblast target cell line. Murine L-929 fibroblast cells (ATCC CCL 1.2) are grown in Eagle's Minimum Essential Medium (MEM) with Earle's salts, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycinfungizone solution, at 37° C. (5% CO_2). A master stock of the cell culture is stored in liquid nitrogen. Working stocks are subcultured twice weekly and maintained for 30 passages. 96-well flat-bottomed trays containing confluent monolayers of the L-929 cells are prepared by adding 75 μl of 2.6×10^5 trypsinized cells/ml to each well and then incubating the plates for 18 hours at 37° C. (5% CO_2). Cell monolayers are prepared one day prior to sample addition.

Samples are serially diluted in a separate dilution plate and transferred to the cell plates to which actinomycin-D has been added to a final concentration of 1 μg/ml immediately prior to sample transfer. Cytotoxicity is scored the following day by spectrophotometrically measuring neutral red dye uptake by viable cells. One unit of TNF activity is defined as that amount required for 50% cell killing. This actinomycin-D enhanced cytotoxicity assay is adapted from those described by J. M. Ostrove and G. E. Gifford in *Proc. Soc. Exp. Biol. Med.*, 160:354–358 (1979) and M. R. Ruff and G. E. Gifford in *Inf. Imm.*, 31:380–385 (1981). Scoring cytotoxicity using neutral red staining is modified from the procedure described by F. C. Kull and P. Cuatrecasas in *J. Immunol.*, 126:1279–1283 (1981).

TNF activity of the sample is determined in comparision to a TNF standard prepared from the final purification product of Examples XI, XII, XIII or XIX. To prepare the TNF standard, TNF final purification product is diluted with MEM Earl's salts medium containing 2% FBS and 1% penicillin-streptomycin-fungizone solution. The diluted material is aliquoted and stored at −70° C. The L-929 cytotoxicity assay, run on at least six different days, is used to titer the standard, setting 1 unit TNF/ml as the amount of TNF producing 50% cell killing.

As a control, a preparation of TNF is aliquoted into vials, and stored at −70° C. In each assay, a vial is assayed along with the other samples for the purpose of evaluating inter-assay variability.

The quantitative measurement of the TNF activity of a sample is performed as follows:

The sample is diluted in assay medium (MEM Earl's salts medium containing 2% FBS and 1% penicillin-streptomycin-fungizone solution) to estimated TNF concentrations between 10^4 and 10^5 units/ml.

An aliquot of the TNF control sample is diluted in assay medium to estimated TNF concentrations between 10^4 and 10^5 units/ml.

96-well trays are filled with 120 μl/well assay medium. 60 μl of one of the following TNF solutions is added to the first well of each row and is serially diluted 1:3 down each row:

a. The sample to be assayed.
b. TNF control sample.
c. In-house TNF standard.

The sample plates are UV sterilized for 10 minutes and then incubated for approximately 10 minutes at 37° C. (5% CO_2).

25 μl/well of actinomycin-D (1 μg/ml final concentration) is added to 96-well flat-bottomed trays containing confluent monolayers of L-929 cells (75 μl), and within two hours 100 μl /well of the serially diluted samples is added. Assay plates are incubated for 18 hours at 37° C. (5% CO_2) and the cells are stained with 50 μl/well of neutral red (0.075%)/glutaraldehyde (10.17%) solution. Plates are incubated for one hour at 37° C. (5% CO_2) and excess staining solution is removed. Plates are washed with 600 μl /well of PBS. 100 μl/well of stain solubilizing solution (3% SDS, 0.04 N HCl in 2-propanol) is added and the plates are shaken for one minute. Absorbance is read at 550 nm using a plate reader.

From the dilution factor required to each 50% cell killing, plate-to-plate variation is corrected by means of the in-house TNF standard. The cytotoxicity activity in the pre-diluted samples is calculated and expressed as units/ml. If the sample was diluted the activity is multiplied by the pre-dilution factor to obtain the units/ml in the sample.

TNF concentration and specific activity can be determined with this information and the protein concentration obtained by standard Lowry test.

EXAMPLE XVI

Protein Purity Assay:SDS-PAGE

To determine the purity of the TNF protein, the final purification product is analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by molecular sizing. Quantitative measurement of protein impurities is obtained from Fast Green-stained gels. The qualitative but highly sensitive evaluation of impurities is obtained from silver-stained gels. The quantitative measurement of protein impurities in the final purification product is performed on

both reducing and non-reducing gels using Fast Green-staining as follows:

For reducing gels, TNF final purification product is diluted in buffered 2-mercaptoethanol solution (10% mercaptoethanol, 18% glycerol, 4% SDS and 0.11 M Tris pH 6.8) to a concentration of about 0.25 mg/ml. This protein solution is heated for three minutes at approximately 95° C. Calibration proteins are treated in an identical manner.

For non-reducing gels, TNF final purification product is diluted in buffered solution (20% glycerol, 4% SDS and 0.12 M Tris pH 6.8) to a concentration of about 0.25 mg/ml and this protein solution is heated for three minutes at approximately 95° C.

The SDS-PAGE apparatus (Hoeffer Scientific Instruments, Model SE-40) is set up containing a 1.5 mm thick gel of a linear 10–15% acrylamide gradient. Approximately 7 μg protein/gel lane (at least 2 lanes per sample), is loaded and electrophoresed. After electrophoresis, the gel slab is stirred in a solution containing 1% Fast Green and 7% acetic acid. The gel is destained with a solution containing 5% methanol and 7% acetic acid and scanned on a densitometer at wavelength 635 nm.

The area of the main peak (assignable to TNF) is measured, divided by the total area measured in the scan (areas due to inclusions in the gel or scratches on the gel carrier plate are subtracted from the total area measurement) and multiplied by 100, to determine the percent of the staining area that is attributable to TNF.

The qualitative evaluation of impurities in the final purification product is performed on both reducing and non-reducing gels using silver-staining. Gels 0.75 mm thick of 15% acrylamide are used. Approximately 1 μg of protein is loaded into a lane. After electrophoresis, silver-staining is accomplished by placing the gel slab in a fixing solution of ethanol/acetic acid, then in a staining solution of silver nitrate/sodium hydroxide-ammonium hydroxide, and finally in a developing solution of formaldehyde/citric acid. The gels are then photographed. A representative silver stained gel is shown as FIG. 2.

EXAMPLE XVII

Protein Purity Assay - Isoelectric Focusing

To determine the purity of the TNF protein, the product also is analyzed by isoelectric focusing (IEF), which separates proteins by their isoelectric point (pI). The qualitative but sensitive evaluation of protein species is obtained from Coorassie Blue-stained gels. The measurement of protein species in the product is performed on polyacrylamide gels as follows:

The IEF flat bed apparatus FBE 3000 (Pharmacia) is set up with a pH 4.0–6.5 Ampholine polyacrylmide gel (PAG) plate and a pH 3.5–9.5 Ampholine PAG plate. Approximately 3 μg protein is loaded per gel lane for each sample and 10 μg/gel lane for the pI standards.

The sample is electrofocused and after electrofocusing, the gels are placed in a fixing solution containing 3.8% 5-sulfosalicylic acid, 12% trichloroacetic acid and 30% methanol. The gels are washed with a solution containing 25% ethanol and 8% acetic acid and stained in a solution containing 0.09% Coomassie Brilliant Blue R, 25% ethanol and 8% acetic acid.

The gels are destained with a solution containing 25% ethanol and 8% acetic acid.

EXAMPLE XVIII

The Limulus Amebocyte Lysate (LAL) test, as described in USP XX, page 888, is used to assess the level of endotoxin present in the final purification product. Lyophilized preparations of lysate and control standard endotoxin are obtained from licensed vendors for use in the test. The LAL test on the final purification product is performed as follows:

Final purification product is suspended in Sterile Water for Injection, U.S.P. Four replicate 2-fold dilution series for the suspended product and for the control standard endotoxin using Sterile Water for Injection, U.S.P. as diluent are prepared. Negative controls consisting of the sterile water diluent only and positive controls consisting of the same diluent inoculated with endotoxin at a level of not more than two times the stated lysate sensitivity are included.

Lysate is added to each tube and the tube is incubated at 37°±1° C. for 60±2 minutes and read. The concentration of endotoxin in the sample is calculated by the formula $(p\lambda)\ (f/\Sigma)$ as described in USP XX.

For an acceptable test, the following conditions must be met:

The lysate sensitivity obtained in the test must be within one serial dilution of the labeled sensitivity. The negative control must exhibit no gelation and firm gelation must occur in the positive control(s).

EXAMPLE XIX

The product of Example XIV is diluted in 20 mM sodium phosphate pH 7.5 concentration sufficient to yield a predetermined specific activity when mixed with a solution of 20% mannitol. 20% mannitol is added and the formulated product is prefiltered through a 0.45 μM filter. The formulated product is lyophilized.

EXAMPLE XX

The desalted product of Example XV is obtained and formulated as in Example XIX.

It will be readily appreciated by those skilled in the art that the processes and compositions according to the invention may be varied without departing from the essence of the invention as disclosed and claimed. Such variations to the process or the compositions obtained thereby are intended to be within the scope of the invention.

It further will be readily appreciated by those skilled in the art that the above-described purified TNF may be formulated with any one of a number of well known pharmaceutically acceptable carriers, depending upon the optical route of administration, e.g., parenteral, including intravenous, intraperitoneal, intramuscular and subcutaneous. Such carriers include solutions compatible with the mode of administration and solubility of the compounds. Such solutions may be buffered or otherwise formulated to minimize undesirable localized effects of injection if necessary.

For parenteral use, the compounds of this invention can be formulated with sterile ingredients compounded and packaged aseptically. They may be administered intravenously or intramuscularly. Useful solvents for formulation in such use are the polyhydric aliphatic alcohols and mixtures thereof. Pharmaceutically acceptable glycols, such as propylene glycols, and mixtures thereof, or glycerine may be employed. Pharmaceutically acceptable sugar alcohols such as mannitol or

sorbitol may be used. Water may be incorporated in the vehicle if desired.

A pH range of about 7.4 and isotonicity compatible with body isotonicity, are desirable. Basicity may be controlled by the addition of a base as required. It may often be desirable to incorporate a local anesthetic, and such are well known to those skilled in the art. The percentage of the compound to be used in the pharmaceutical carrier may be varied. It is necessary that the compound constitute a proportion such that a suitable dosage will be obtained.

The dosage required to achieve the desired pharmacologic activity in the mammal will vary with various factors such as route of administration, the species of mammal, general health and tolerances of the mammal, weight, sex and age of the mammal, the nature and severity of the disease being treated and the like. Additionally, it is to be noted that the exact dosage of each individual compound employed in similar situations will vary.

What is claimed is:

1. A purified TNF composition wherein the TNF is N-terminally deleted, lacking from 1 to 10 amino acids, and is substantially free of pyrogens as determined by the USP rabbit pyrogen test at a dosage range of about 1.0 to 2.4×10^5 U/Kg, and is substantially non-bindable to a hydrophobic porous membrane.

2. The purified TNF composition of claim **1** wherein the N-terminally deleted TNF is selected from the group consisting of minus 1 through minus 10 TNF.

3. The purified TNF composition of claim **2** wherein the TNF is minus 8.

4. The purified TNF composition of claim **2** wherein the TNF is minus 7.

5. The purified TNF composition of claim **2** wherein the TNF is minus 6.

6. The purified TNF composition of claim **2** wherein the TNF is minus 4.

7. The purified TNF composition of claim **2** wherein the TNF is minus 2.

8. The purified TNF composition of claim **1** wherein said hydrophobic porous membrane is constructed of materials selected from the group consisting of polytetrafluroethylene, polypropylene and polyethylene.

9. The purified TNF composition of claim **1** wherein said porous hydrophobic membrane consist of polytetrafluroethylene.

* * * * *

United States Patent [19]

Mandell et al.

[11] **Patent Number: 4,965,271**

[45] **Date of Patent: Oct. 23, 1990**

[54] **METHOD OF INHIBITING THE ACTIVITY OF LEUKOCYTE DERIVED CYTOKINES**

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[21] Appl. No.: **131,785**

[22] Filed: **Dec. 11, 1987**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 947,905, Dec. 31, 1986, abandoned.

[51] **Int. Cl.**[5] **A61K 31/52**

[52] **U.S. Cl.** **514/263;** 514/929

[58] **Field of Search** 514/263, 929

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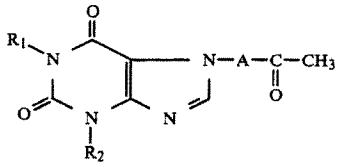
Attorney, Agent, or Firm—Finnegan, Henderson, Farabow, Garrett & Dunner

[57] **ABSTRACT**

A family of compounds effective in inhibiting interleukin-1 (IL-1) activity, tumor necrosis factor (TNF) activity, and the activity of other leukocyte derived cytokines is comprised of 7-(oxoalkyl) 1,3-dialkyl xanthines of the formula



(I)

in which R_1 and R_2 are the same or different and are selected from the group consisting of straight-chain or branched alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, alkoxyalkyl and hydroxyalkyl radicals, and A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group. The inhibition of Il-1, TNF, and other cytokines in mammals is implicated in allerviation of a wide variety of disease conditions.

14 Claims, 7 Drawing Sheets

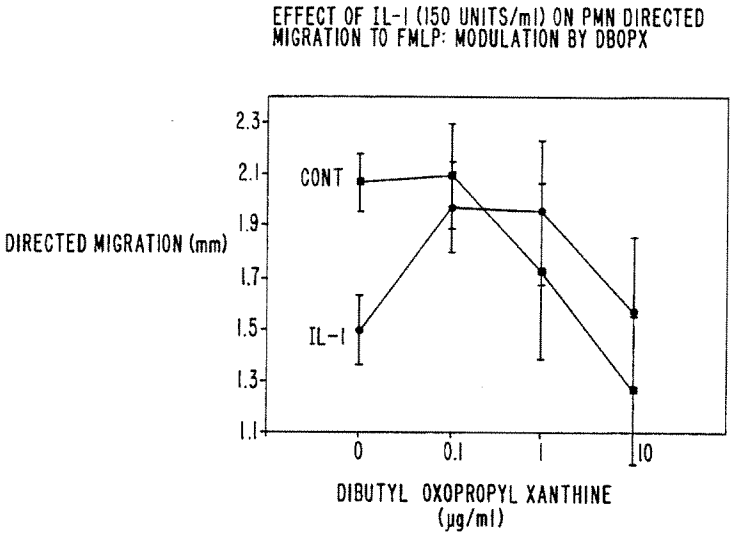


FIG. 1

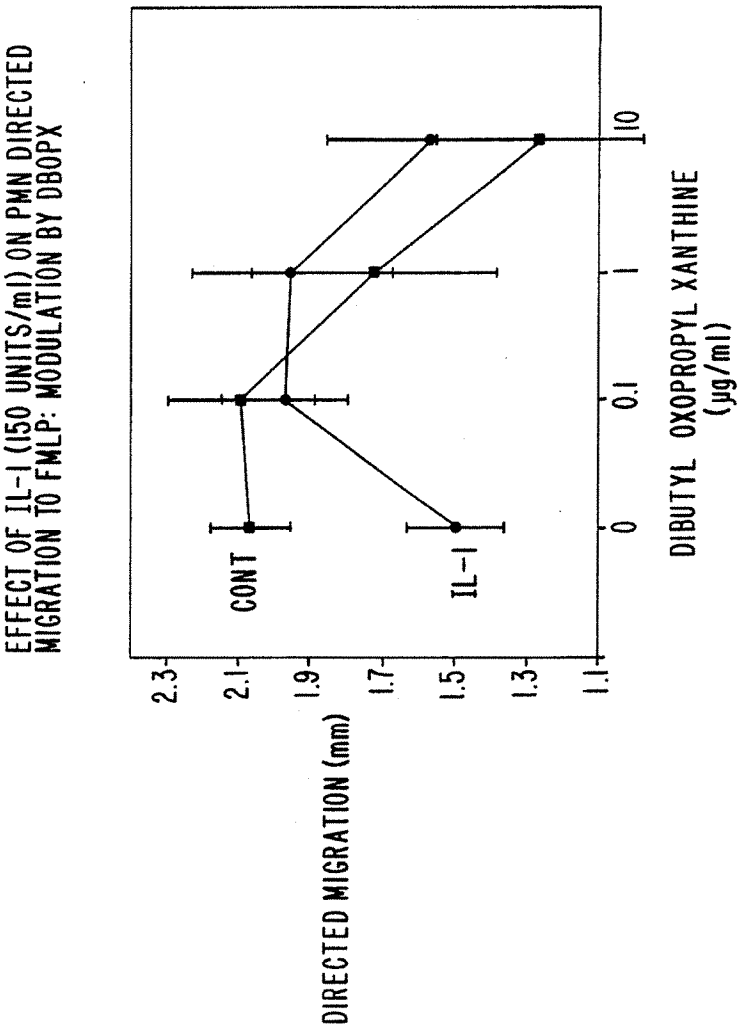

EFFECT OF IL-1 (150 UNITS/ml) ON PMN DIRECTED MIGRATION TO FMLP: MODULATION BY DBOPX
DIRECTED MIGRATION (mm)
2.3
2.1
1.9
1.7
1.5
1.3
1.1
CONT
IL-1
0
0.1
1
10
DIBUTYL OXOPROPYL XANTHINE (µg/ml)

FIG. 2

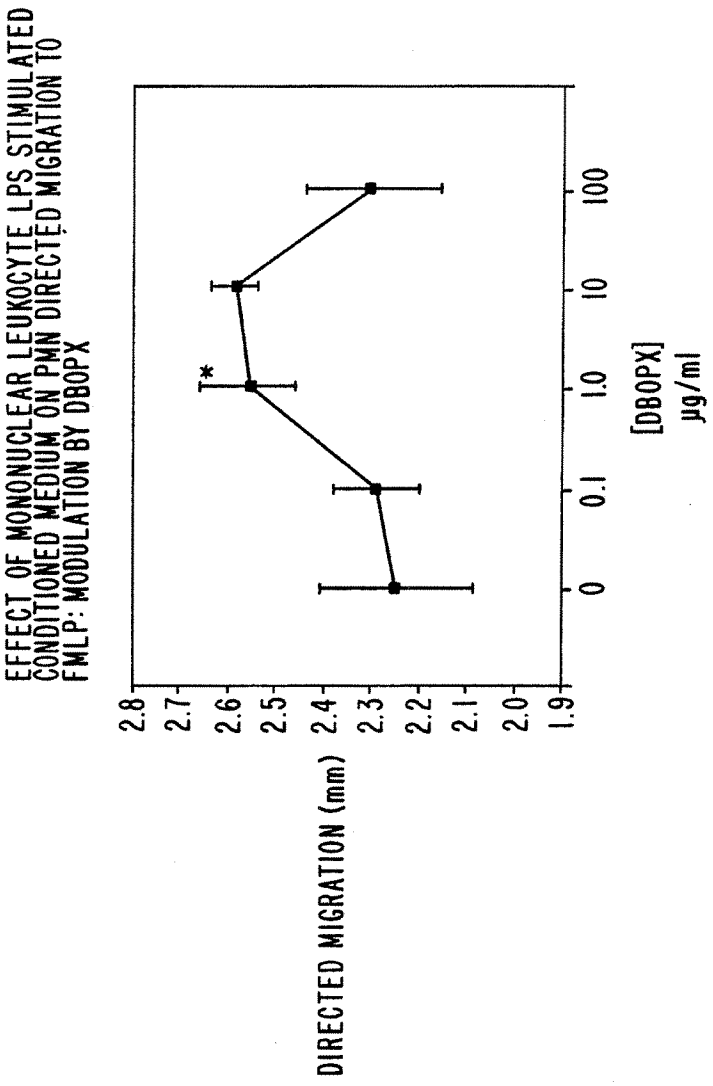

EFFECT OF MONONUCLEAR LEUKOCYTE LPS STIMULATED CONDITIONED MEDIUM ON PMN DIRECTED MIGRATION TO FMLP: MODULATION BY DBOPX
DIRECTED MIGRATION (mm)
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
*
0
0.1
1.0
10
100
[DBOPX] µg/ml

FIG. 3

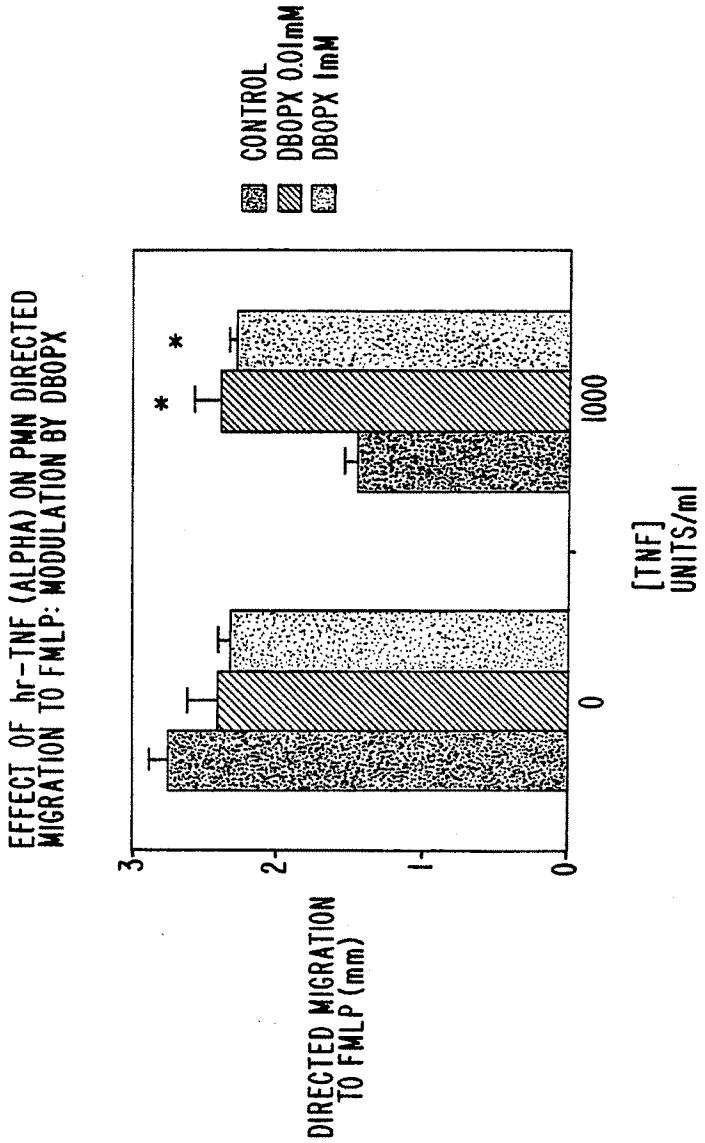

EFFECT OF hr-TNF (ALPHA) ON PMN DIRECTED MIGRATION TO FMLP: MODULATION BY DBOPX
CONTROL
DBOPX 0.01mM
DBOPX 1mM
DIRECTED MIGRATION TO FMLP (mm)
3
2
1
0
0
1000
[TNF] UNITS/ml
*
*

FIG. 4

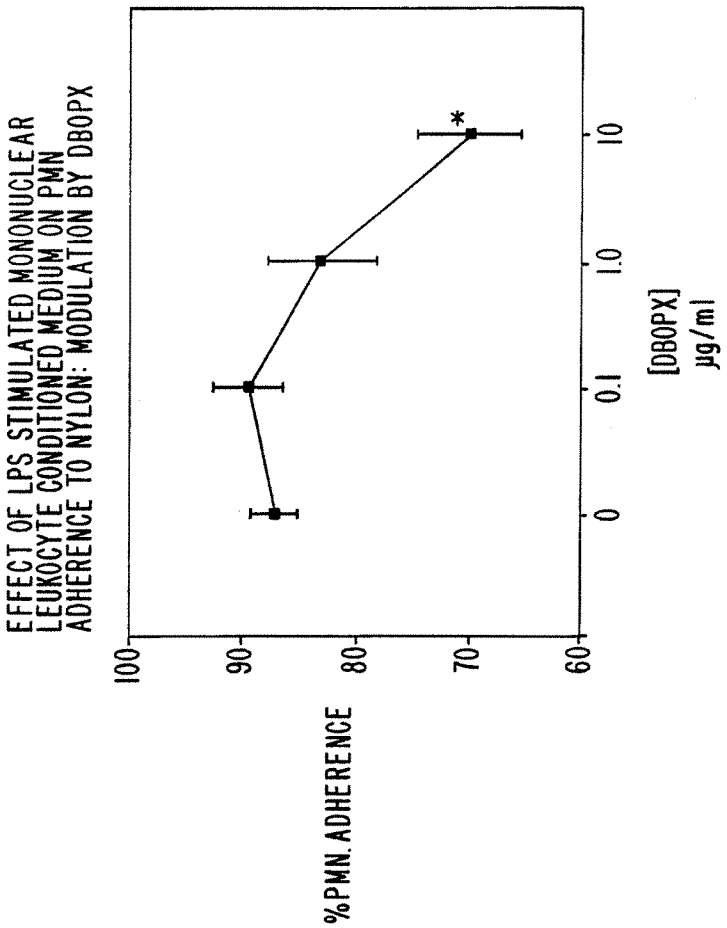

EFFECT OF LPS STIMULATED MONONUCLEAR LEUKOCYTE CONDITIONED MEDIUM ON PMN ADHERENCE TO NYLON: MODULATION BY DBOPX
%PMN. ADHERENCE
100
90
80
70
60
0
0.1
1.0
10
[DBOPX] µg/ml
*

FIG. 5

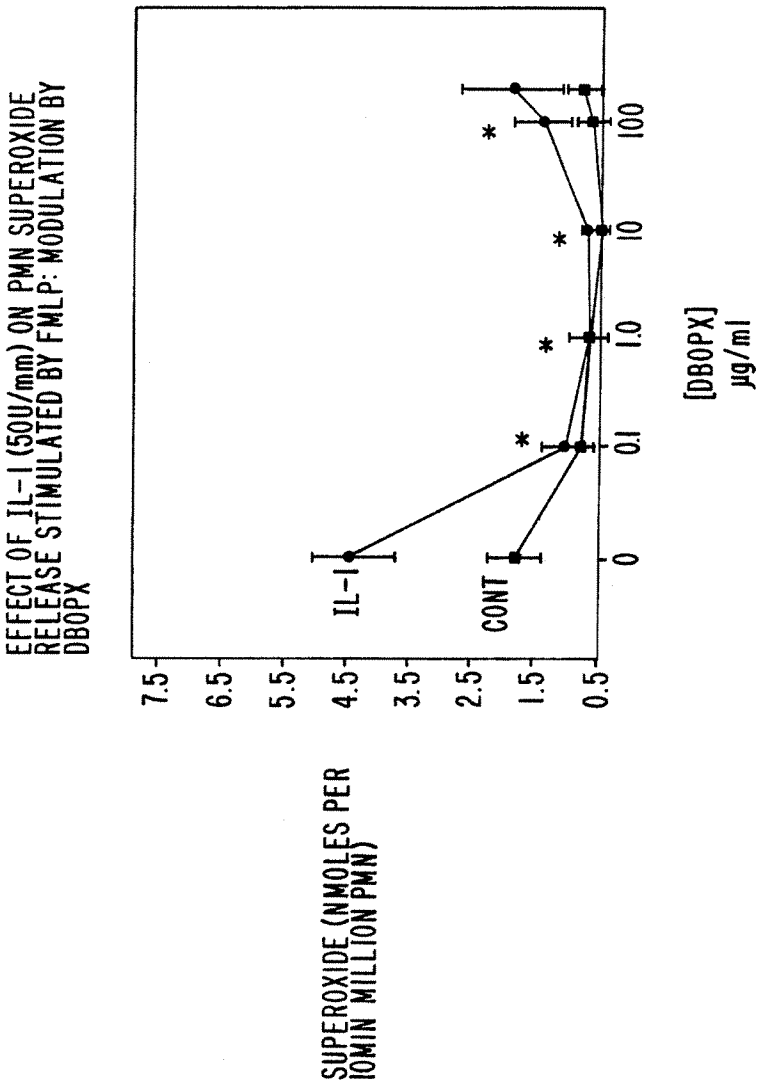

EFFECT OF IL-1 (50U/mm) ON PMN SUPEROXIDE RELEASE STIMULATED BY FMLP: MODULATION BY DBOPX
SUPEROXIDE (NMOLES PER 10MIN MILLION PMN)
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
IL-1
CONT
*
*
*
*
0
0.1
1.0
10
100
[DBOPX] µg/ml

FIG. 6

THE EFFECT OF LPS STIMULATED MONONUCLEAR LEUKOCYTE CONDITIONED MEDIUM ON SUPEROXIDE PRODUCTION BY PMN STIMULATED WITH FMLP (100nM): MODULATION BY DBOPX

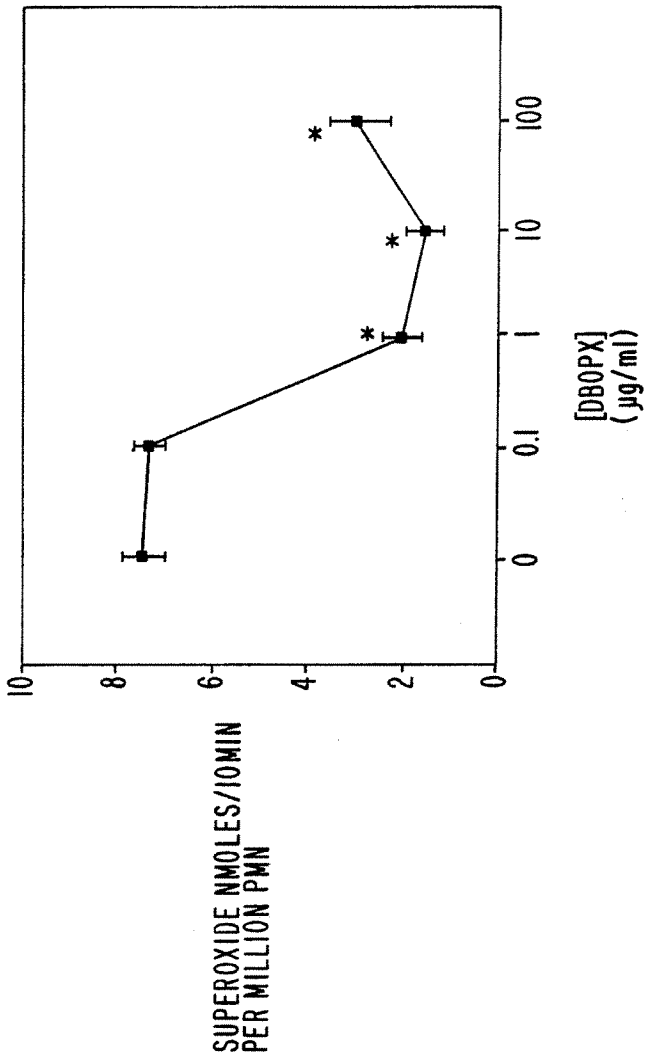
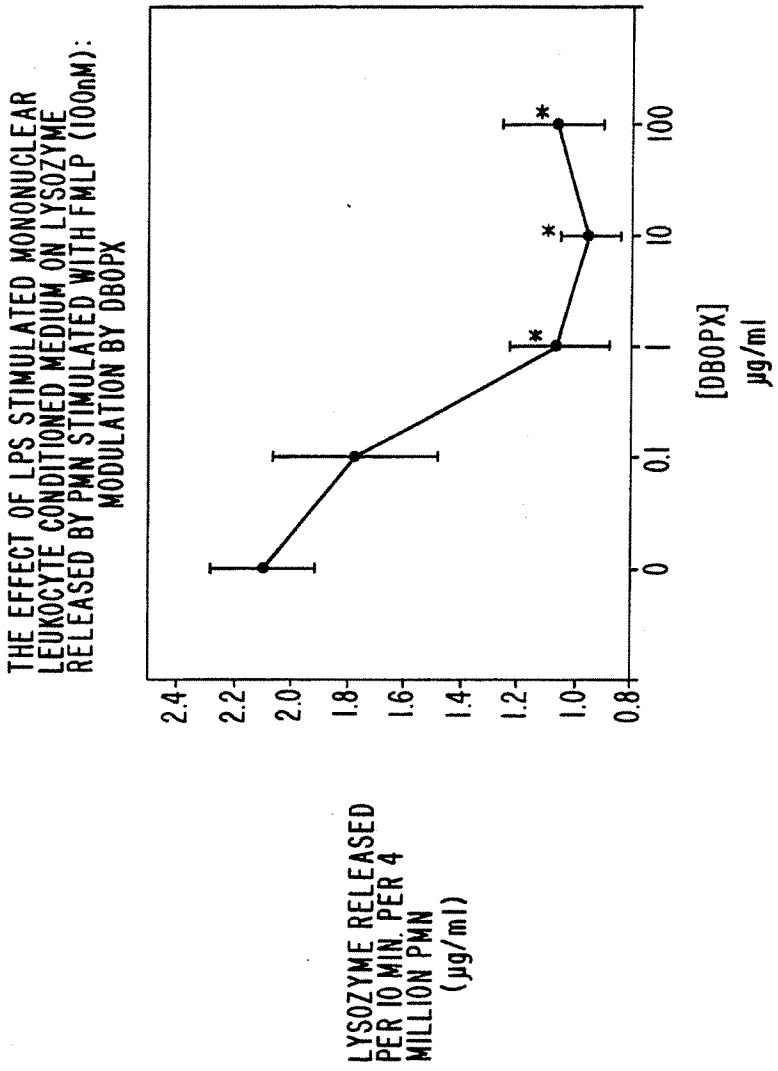


FIG. 7


THE EFFECT OF LPS STIMULATED MONONUCLEAR LEUKOCYTE CONDITIONED MEDIUM ON LYSOZYME RELEASED BY PMN STIMULATED WITH FMLP (100nM): MODULATION BY DBOPX
LYSOZYME RELEASED PER 10 MIN. PER 4 MILLION PMN (µg/ml)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
0.1
1
10
100
[DBOPX] µg/ml
*
*
*

METHOD OF INHIBITING THE ACTIVITY OF LEUKOCYTE DERIVED CYTOKINES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending application Ser. No. 947,905, filed Dec. 31, 1986, now abandoned for METHOD OF INHIBITING INTERLEUKIN-1 ACTIVITY AND THAT OF OTHER LEUKOCYTE DERIVED CYTOKINES, by Gerald L. Mandell, Gail W. Sullivan, and William J. Novick. The entire disclosure of the related, copending application is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

This invention relates to the inhibition of activity of leukocyte derived cytokines, such as interleukin-1 and tumor necrosis factor, in humans and mammals. More specifically, this invention provides a method of inhibiting the activity of cytokines to arrest or alleviate certain disease and inflammatory states.

Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are biological substances produced by monocytes and other macrophages in mammals. IL-1 and TNF affect a wide variety of cells and tissues, both in vitro and in vivo. Research has demonstrated that IL-1, TNF, and other leukocyte derived cytokines are important, and even critical, mediators in a wide variety of inflammatory states and diseases. The inhibition of IL-1, TNF, and other leukocyte derived cytokines is of benefit in controlling, reducing, and alleviating many of these conditions.

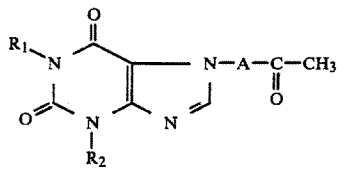
Detection on and inhibition of IL-1, TNF, and other leukocyte derived cytokines can be relatively easily documented through in vitro analysis of polymorphonuclear neutrophil behavior. Among other activities attributed to IL-1 and other leukocyte derived cytokines is the promotion of leukocyte adherence and the inhibition of neutrophil chemotaxis, both directly contributing to disease and inflammation syndromes.

Despite the desirability of inhibiting the activity of IL-1 and TNF and the activity of other leukocyte derived cytokines and the ease with which inhibition can be detected in vitro, there exists a need in the art for inhibitors of IL-1, TNF, and other cytokines, wherein the inhibitors are acceptable for in vivo administration.

SUMMARY OF THE INVENTION

This invention aids in fulfilling these needs in the art by identifying a class of compounds that can be successfully employed in alleviating conditions caused by, or mediated by, IL-1, TNF, and other leukocyte derived cytokines. The compounds exhibit marked inhibition of cytokine activity, even at low concentrations of the mediators as demonstrated through in vitro tests.

More particularly, this invention provides a method of inhibiting the activity of IL-1, TNF, and other leukocyte derived cytokines in a mammal comprising administering thereto at least one 7-(oxoalkyl) 1,3-dialkyl xanthine of the formula



(I)

in which

R_1 and R_2 are the same or different and are independently selected from the group consisting of straight-chain or branched-chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl, and hydroxyalkyl radicals; and

A is a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group. The xanthine is employed in an amount that is effective in inhibiting the activity of IL-1, TNF, and other leukocyte derived cytokines in the mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be more fully described with reference to the drawings in which:

FIG. **1** is a graph showing modulation by 1,3-dibutyl 7-(2-oxopropyl) xanthine (DBOPX) of the effect of interleukin-1 (IL-1) on polymorphonuclear leukocyte (PMN) directed migration to n-formyl methionyl leucyl phenylalanine (FMLP);

FIG. **2** shows the results of modulation by DBPOX of the effect of mononuclear leukocyte LPS stimulated conditioned medium on PMN directed migration to FMLP;

FIG. **3** shows the results of modulation by DBOPX of the effect of tumor necrosis factor (TNF) on PMN directed migration to FMLP;

FIG. **4** shows the results of modulation by DBOPX of LPS stimulated mononuclear leukocyte conditioned medium on PMN adherence to nylon;

FIG. **5** shows the results of modulation by DBOPX of IL-1 on PMN superoxide release stimulated by FMLP;

FIG. **6** is a graph showing modulation by DBOPX of lipopolysaccharide (LPS) stimulated mononuclear leukocyte conditioned medium on superoxide production by PMN stimulated with FMLP; and

FIG. **7** is a graph showing modulation by DBOPX of the effect of LPS-stimulated mononuclear leukocyte conditioned medium on lysozyme released by PMN stimulated with FMLP.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

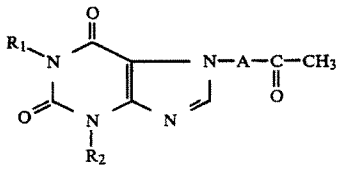
Inhibition of the activity of IL-1, TNF, and other leukocyte derived cytokines can be achieved by the administration of 7-(oxoalkyl) 1,3-dialkyl xanthines to a mammal.

As used herein, the expression "leukocyte derived cytokines" is to be given a broad meaning. Specifically, the term "leukocyte" as used herein means mammalian cells of granulocytic and lymphocytic lineage. Examples of leukocyte cells are polymorphonuclear leukocytes, such as neutrophils, and mononuclear phagocytes, such as monocytes and macrophages and lymphocytes.

The term "cytokine" as used herein means a secretory product of a leukocyte, and in particular a non-

antibody protein released by a leukocyte on contact with antigen and which acts as an intercellular mediator of immune response. Examples of cytokines that are within the scope of this invention are chemotactic factors, factors promoting replication of lymphocytes, factors inhibiting replication of lymphocytes, factors affecting macrophage adherence, factors affecting enzyme secretion by macrophages, and factors that mediate secretion of oxidizing agents, such as oxygen, superoxide, hydrogen peroxide and hydroxyl radical.

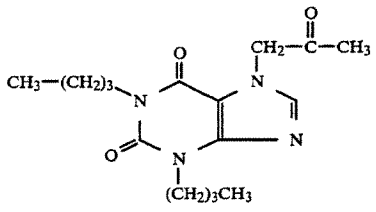
The 7-(oxoalkyl)1,3-dialkyl xanthines employed in this invention have the following formula:



(I)

The substituents R_1 and R_2 in formula (I) are the same or different and are independently selected from the group consisting of straight-chain or branched alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, alkoxyalkyl and hydroxyalkyl radicals. The substituent A represents a hydrocarbon radical with up to 4 carbon atoms, which can be substituted by a methyl group.

A compound that has been found to be particularly effective for inhibiting the effects of IL-1 and other leukocyte derived cytokines on polymorphonuclear leukocytes and monocytes is 1,3-dibutyl 7-(2-oxopropyl) xanthine. This compound, which is also referred to herein in abbreviated form as "DBOPX", has the following formula:



(II)

"DBOPX"

The ability of compound (II) to inhibit the effects of IL-1 and other leukocyte derived cytokines on polymorphonuclear leukocyte and monocyte adherence, cell chemotaxis, respiratory (metabolic) burst, and cell degranulation has been demonstrated and is described hereinafter.

Phagocytes important in immunology are polymorphonuclear leukocytes (e.g. neutrophils) and mononuclear phagocytes (e.g. monocytes and macrophages). Phagocyte hypofunction is a cause of recurrent pyogenic infection. To combat pyogenic infection, neutrophils and monocytes respond to chemotactic factors by moving toward the source of infection, where they ingest microorganisms and kill them.

More particularly, a main function of polymorphonuclear leukocytes and monocyte is to kill bacteria and other infectious agents by phagocytosis. The first stage in the ingestion and digestion of a particulate substance by these cells involves the process of bringing the cells and the particles together, usually through chemotaxis. This response is an essential part of host defense against infection. The extensive migration and activity of these cell is manifested by inflammation at the site of injury or invasion of the host.

It has been shown that IL-1 and TNF inhibit chemotaxis by granulocytes, monocytes and macrophages. It has now been discovered that the 7-(oxoalkyl)1,3-dialkyl xanthines of formula (I are capable of modulating the inhibitory effect of IL-1 and TNF on chemotaxis. This has been demonstrated as follows.

The migration of polymorphonuclear leukocytes in response to n-formyl methionyl leucyl phenylalanine (FMLP), a well known chemotactic factor, was determined by chemotaxis under agarose, a well known assay for cell chemotaxis. See *J. of Immunol.*, 115, 6, 1650–1656 (1975). The assay was carried out without IL-1, and the assay was repeated in the presence of IL-1. The assay was also carried out with IL-1, but without DBOPX, and with both IL-1 and DBOPX at DBOPX concentrations of 0.1, 1, and 10 micrograms per milliliter (μg/ml). The results are depicted in FIG. **1**.

As shown in FIG. **1**, directed migration of the cells in the absence of IL-1, TNF, and with 0 μg/ml DBOPX (i.e. "CONT" in FIG. 1) was about 2.08 mm. Directed migration of the cells dropped to about 1.5 mm in the presence of IL-1, TNF, and with 0 μg/ml DBOPX. Thus, IL-1 inhibited cell chemotaxis directed to FMLP.

FIG. **1** also shows the effect of increasing concentrations of DBOPX on the inhibition of chemotaxis by IL-1. More particularly, DBOPX modulates the inhibitory effect of IL-1 on directed migration to FMLP. Specifically, FIG. **1** shows that DBOPX increased directed migration of the cells and modulated the inhibitory effect of IL-1 at all of the DBOPX concentrations that were evaluated. FIG. **1** also shows that DBOPX was effective in increasing chemotaxis even at very low DBOPX concentrations. Thus, the compounds employed in the process of this invention are particularly effective in modulating the inhibitory effect of IL-1 on cell chemotaxis.

DBOPX is capable of producing a similar effect on polymorphonuclear leukocytes incubated with the products of mononuclear leukocytes that were stimulated with lipopolysaccharide (LPS). These mononuclear cells produce IL-1, TNF, and other inflammatory cytokines. Once again, polymorphonuclear leukocyte directed migration to FMLP was determined by chemotaxis under agarose. The assay was carried out without DBOPX and with concentrations of DBOPX of 0.1, 1.0, 10, and 100 μg/ml. The results are shown in FIG. **2**.

Referring to FIG. **2**, the directed migration of the PMN in the conditioned medium containing the inflammatory cultures was about 2.25 mm in the absence of DBOPX. The addition of DBOPX to the medium increased directed migration of the cells at all of the DBOPX concentrations tested. Once again, DBOPX was effective in increasing chemotaxis even at very low concentrations. Moreover, the directed migration was about 2.6 mm at a DBOPX concentration of 10 μg/ml. By comparison, migration in an unconditioned medium containing LPS was 2.60±0.5mm. (Data not shown in FIG. **2**). The probability that DBOPX increased directed migration inhibited by conditioned medium containing inflammatory cultures was 95%.

DBOPX is capable of producing a similar effect on PMN incubated with rh-TNF (alpha). PMN directed

migration to FMLP was determined by chemotaxis under agarose. The assay was carried out without DBOPX and with concentrations of DBOPX of 0.01 mM (3.2 μg/ml) and 1 mM (320 μg/ml). The results are shown in FIG. 3.

Referring to FIG. 3, the directed migration of the PMN in medium containing rh-TNF was 1.45 mm in the absence of DBOPX. The addition of DBOPX to the medium increased directed migration of the cells at both of the DBOPX concentrations tested. Once again, DBOPX was effective in increasing chemotaxis even at very low concentrations. By comparison, migration in medium in the absence of TNF was 2.75 mm. The probability that DBOPX increased directed migration inhibited by TNF was better than 95%.

Thus, the 7-(oxoalkyl) 1,3-dialkyl xanthines employed in the process of invention are capable of increasing directional movement of polymorphonuclear leukocytes. These compounds can be administered to a patient to augment chemotactic factors of bacterial or viral origin, or components of plasma activation systems, or factors elaborated by cells of the immune system.

Leukocyte response to an acute inflammatory stimulus involves a complex series of events, including adherence to endothelium near the stimulus. Inhibition of leukocyte adherence can be expected to reduce the degree of inflammation seen in conditions, such a septic shock and adult respiratory distress syndrome. It has been found that the 7-(oxoalkyl) 1,3-dialkyl xanthines employed in this invention effectively block adherence of polymorphonuclear leukocytes.

Specifically, polymorphonuclear leukocyte (PMN) adherence to nylon was determined according to the method of MacGregor et al.

New Engl. J. Med. 13:642–646 (1974). Purified PMN cells were incubated with a lipopolysaccharide-stimulated mononuclear leukocyte conditioned medium containing inflammatory cytokines. PMN adherence to nylon was determined without DBOPX, and then with DBOPX at concentrations of 0.1, 1.0, and 10 μg/ml. The percent PMN adherence to nylon was determined for each case. The results are summarized in FIG. 4.

FIG. 4 shows that PMN adherence to nylon in the absence of DBOPX was about 87%. However, when DBOPX was included in the assay at concentrations above about 0.1 μg/ml, PMN adherence to the nylon was inhibited as evidenced by a decline in percent adherence. At a DBOPX concentration of 10 μg/ml, the percent PMN adherence declined to about 70%. The probability that DBOPX decreased adherence of PMN incubated with conditioned medium was 99.7%. Thus, the compounds employed in the process of this invention are particularly effective in blocking adherence of leukocytes and thereby aiding in reducing the degree of inflammation.

Mature phagocytes are in a metabolically dormant state. It is currently believed that recognition of certain objects and substances by phagocytes, such as the attachment of an ingestible particle to the cell surface, changes this situation, and the cell enters a stage of increased metabolic activity, which is referred to as metabolic or respiratory burst. The transition is associated with a series of characteristic changes, including the production of a superoxide anion. Cytokines, such as IL-1 and TNF, are capable of producing a similar effect. In addition to its significance for phagocytic function related to inactivation of ingested microbes, activation of oxygen metabolism is a useful indirect marker for the ingestion process per se. It would be desirable to be able to modulate the effect of cytokines on respiratory burst.

Quantitative methods for direct measurement of hydrogen peroxide and superoxide anions released into the medium are currently available. It has been found that the compounds employed in this invention are capable of modulating respiratory burst in stimulated polymorphonuclear leukocytes (PMN) as determined using these methods.

More particularly, superoxide production was assayed using a modification of the procedure described by Babior et al., *J. Clin. Investigation*, 52:741–744 (1973). Purified PMN were incubated with an oxidative stimulus with and without IL-1. The medium was assayed for superoxide production. The assay was also carried out without DBOPX and with DBOPX in concentrations of 0.1, 1.0, 10, and 100 μg/ml. The results are shown in FIG. 5.

It is evident from FIG. 5 that about 1.8 nmoles of superoxide/10 min/million PMN were produced by FMLP-stimulated PMN in the absence of IL-1, TNF, and DBOPX (see "CONT" in FIG. 5). Pretreatment with IL-1 (5 units/20 μl), which is known as priming, produced a substantial increase in observed superoxide release to about 4.4 nmoles superoxide/10 min/million PMN.

In contrast, the addition of DBOPX to the assay resulted in a substantial reduction in observed superoxide production as is evident from FIG. 5. Specifically, DBOPX modulated the effect or IL-1 on stimulated PMN at all of the concentrations tested. DBOPX was even effective at a very low concentration of 0.1 μg/ml. The probability that DBOPX decreased superoxide production produced by PMN primed with IL-1, TNF, and stimulated with FMLP compared with IL-1 alone was 95%.

DBOPX is also capable of decreasing superoxide production by PMN primed with LPS-stimulated mononuclear leukocyte conditioned medium containing inflammatory cytokines. This is shown in FIG. 6. Specifically, when PMN were incubated with LPSstimulated mononuclear leukocyte conditioned medium containing inflammatory cytokines and stimulated with FMLP, observed superoxide production in the absence of DBOPX was about 7.4 nmoles/10 min/-million PMN. When DBOPX was added to the assay, however, observed superoxide production was lower at all of the DBOPX concentrations tested. Moreover, DBOPX exhibited some effect even at a concentration as low as 1.0 μg/ml. At a DBOPX concentration of 10 μg/ml, superoxide production was about 1.5 nmoles/10 min/million pMN. The probability that DBOPX decreased superoxide production produced by PMN primed with conditioned medium and stimulated with FMLP was 99.5%.

It is evident from these results that the compounds employed in the process of this invention are capable of reducing superoxide production and modulating respiratory burst in phagocytes, such as polymorphonuclear leukocytes and monocytes.

During ingestion, granules in the cytoplasm of the cell fuse with the membrane of a vacuole that was formed around the foreign substance. The granules discharge their contents into the vacuole. Some of this material ends up in the medium surrounding the phagocyte. Since the granules disappear during this process, it

is called degranulation. The granule contents include hydrolytic enzymes, lysozyme, bactericidal proteins, and, in the neutrophil, myleoperoxidase.

Degranulation can be assessed by measuring the rate of appearance of granule-associated enzymes in the extracellular medium. In the case of polymorphonuclear leukocytes (PMN), degranulation can be assayed by determining release of lysozyme. It was found that the compounds employed in the process of this invention are capable of modulating the release of lysozyme from stimulated PMN.

More particularly, polymorphonuclear leukocytes (PMN) were incubated with LPS-stimulated mononuclear leukocyte conditioned medium containing inflammatory cytokines. The PMN were then stimulated with FMLP, incubated for a period of time, and lysozyme content was determined in cell supernatant using a well known assay. See *J. Bacteriol.*, 58, 731-736 (1949) The PMN were incubated without DBOPX or with DBOPX in a concentration of 0.1, 1, 10, or 100 μg/ml. The results, which are expressed in terms of lysozyme released/10 min/4 million PMN (μg/ml), are shown in FIG. 7.

Referring to FIG. 7, lysozyme released by PMN primed with LPS-stimulated mononuclear leukocyte conditioned medium (containing inflammatory cytokines) and stimulated with FMLP was about 2 1 μg/ml in the absence of DBOPX. When DBOPX was added to the assay, lysozyme release declined. The decrease was observed at all of the concentrations of DBOPX that were evaluated. Moreover, DBOPX was effective in modulating lysozyme release even at concentrations as low as 0.1 μg/ml. At a DBOPX concentration of 100 μg/ml, the lysozyme release was only about 1.04 μg/ml. The probability that DBOPX inhibited lysozyme release from PMN primed with conditioned medium and stimulated with FMLP was 95%.

It is apparent from these results that the compounds employed in the process of this invention are capable of decreasing the release of lysozyme from PMN primed with LPSstimulated mononuclear leukocyte conditioned medium and then stimulated with FMLP.

In summary, the compounds of formula (I) employed in the process of this invention are capable of modulating the effects of leukocyte derived cytokines, such as interleukin-1 and tumor necrosis factor, on phagocytes, such as polymorphonuclear leukocytes. The compounds are capable of substantially aiding chemotaxis. In addition, the compounds can block adherence of cells. The compounds can decrease oxidative damage to host tissues by phagocytes as evidenced by modulation of respiratory burst in stimulated polymorphonuclear leukocytes. Finally, the compounds can modulate the effects of cytokines on degranulation in stimulated phagocytes. The demonstrated inhibition of IL-1, TNF, and other cytokines by these compounds is suggestive of clinical effectiveness in at least the following areas and conditions.

Because IL-1, TNF, and other leukocyte derived cytokines have been implicated in such a wide variety of mammalian conditions, this invention has a similarly broad scope of application. Among the conditions that can be treated or alleviated by the inhibition of IL-1, TNF, and other leukocyte derived cytokines are: sepsis, septic shock, endotoxic shock, gram negative sepsis, toxic shock syndrome, adult respiratory distress, fever and myalgias due to infection (i.e. influenza), cachexia secondary to infection or malignancy, cachexia secondary to AIDS, rheumatoid arthritis, gouty arthritis, osteoporosis, keloid formation, scar tissue formation, decreased appetite, Crohn's disease, ulcerative colitis, fever due to central nervous system bleeding, glomerulonephritis, multiple sclerosis, Creutzfeld-Jacob disease, adverse reactions to dialysis, diabetes melitus, and psoriasis.

By reference to the specific cause of the disease condition, the more generic term "trauma" can be used. The term "trauma" refers broadly to cellular attack by foreign bodies and physical injury of cells. Included among foreign bodies are microorganisms, particulate matter, chemical agents, and the like. Included among physical injuries are mechanical injuries, such as abrasions, lacerations, contusions, wounds, and the like; thermal injuries, such as those resulting from excessive heat or cold; electrical injuries, such as those caused by contact with sources of electrical potential; and radiation damage caused, for example, by prolonged, extensive exposure to infrared, ultraviolet or ionizing radiations.

Microorganisms included among the foreign bodies that can elicit a biological response are bacilli, fungi and yeast, viruses, parasites, and the like. Representative bacilli are: Actinomyces spp.; Bacteroides spp.; Corynebacterium spp.; Enterobacteriacea; Enterococcus; Haemophilus spp.; Micrococcus spp.; Neissera spp.; *Staphylococcus aureus; Streptococcus pneumoniae*; Clostridium spp.; *Streptococrus agalactiae*; Bacillus spp.; *H. influenzae*; Moraxella spp.; Mycobacteria spp.; *Pseudodomonas aeruginosa*; Vibrio spp.; and Mycoplasma.

Representative fungi and yeast that are capable of eliciting a biological response are: Microspurum; Blastomyces; Histoplasma; Aspergillus; Cryptococcus; Candida spp.; Coccidioides; and *Candida albicans.*

Representative viruses are: Rhinovirus; Parainfluenza; Enterovirus; Influenza; Smallpox and vaccinia; Herpes simplex; Measles; Rubella; Arbovirus (Western, Eastern and Venezuelan equine encephalitis, and California encephalitis); Rabies; Colorado tick fever; Yellow fever; Dengue; Hepatitis Virus B (HB Ag); Hepatitis Virus A (HAV); and Human Immunodeficiency Virus (HIV).

Representative parasites that can elicit a response are: Trypanosoma cruzi; Entamoeba histolytica; Leishmania brasiliensis; Leishmania tropica; Leishmania donovani; Toxiplasma gondii; Plasmodium falcipaum; Trypanosoma rhodesiense; Loa loa; Trichomonas hominis; Schistosoma japonicum; Schistosoma mansoni; and Fasciola hepatica.

Particulate materials capable of eliciting a biological response include silica, asbestos, monosodium urate, cotton fibers, coal dust, beryllium, and the like.

Chemical agents include heavy metals, such as lead, chromium, mercury, arsenic, and the like; organic solvents, such as trichloroethylene, and the like; herbicides, such as trichlorophenoxyacetic acid and the like; and pesticides, such as mirex and the like.

In addition, inhibition of IL-1, TNF, and other leukocyte derived cytokines will enhance phagocyte activity in stored blood and blood products.

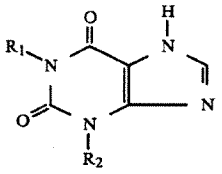
The compounds employed in this invention will now be described in more detail, and methods for preparing the compounds will be provided.

The process of this invention utilizes 7-(oxoalkyl) 1,3-dialkyl xanthines of formula (I) above. While DBOPX is the particularly preferred xanthine, a number of other compounds can be employed. For example,

the xanthines of formula (I) can be substituted by other alkyl groups, or by alkoxy or hydroxyalkyl groups. Suitable alkyl groups include branched and straight chain groups, such as ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, amyl, hexyl, and the like. Alkoxy substituted alkyl groups are branched and straight chain groups containing from 2 to 6 carbon atoms in the combined alkoxy and alkyl groups, including methoxymethyl, amyloxymethyl, methoxyethyl, butoxyethyl, propoxypropyl, and the like. Hydroxyalkyl groups are those containing from 1 to 6 carbon atoms, such as hydroxymethyl, hydroxyethyl, hydroxypropyl, hydroxyhexyl, and the like.

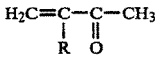
The hydrocarbon group represented by A in formula (I) above are divalent saturated aliphatic hydrocarbon groups, i.e., methylene, ethylene, trimethylene and tetramethylene, which can be substituted on the carbon adjacent the carbonyl group with methyl. Such methyl-substituted groups include ethylidine, 1,2-propylene, and 1,3-butylene groups.

The compounds employed in this invention can be synthesized using known techniques. For example, the compounds can be prepared at elevated temperature, optionally in the presence of a solvent, by reacting correspondingly substituted 1,3-dialkyl xanthines of the formula



(III)

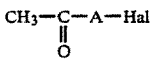
in which R_1 and R_2 are as defined above with α, β-unsaturated methyl ketones corresponding to the formula



(IV)

The substituent R in formula (IV) represents hydrogen or a methyl group. The reaction can be conducted in an alkaline medium.

An alternative method of preparation involves reacting alkali metal salts of 1,3-dialkyl xanthine derivatives of general formula II, in which R_1 and R2 are as defined above, with oxoalkyl halides corresponding to the formula



(V)

in which A is as defined above, and Hal represents a halogen atom, preferably chlorine or bromine.

These reactions are preferably carried out at temperatures in the range from 40° to 80° C., optionally under elevated or reduced pressure, but usually at atmospheric pressure. The individual starting compounds can be employed either in stoichiometric quantities or in excess. The alkali salts in the alternative method of preparation can either be prepared beforehand or in the reaction itself.

Suitable solvents for use in the reactions are water-miscible compounds, preferably lower alcohols, such as methanol, propanol, isopropanol, and various butanols; also acetone; pyridine; triethylamine; polyhydric alcohols, such as ethylene glycol and ethylene glycol monomethyl or monoethyl ether.

The compounds of formula (I) are known for their marked effect in increasing blood flow through skeletal muscle and by their low toxicity. The most active of these compounds for use in accordance with the present invention is 1,3-dibutyl 7-(2-oxopropyl)xanthine, i.e. DBPOX.

A more detailed description of the compounds employed in this invention and methods of preparing the compounds are contained in U.S. Pat. No. 4,242,345, the entire disclosure of which is relied upon and incorporated by reference herein.

Effective amounts of the xanthines can be administered to a subject by any one of various methods, for example, orally as in capsule or tablets, or parenterally in the form of sterile solutions. The xanthines, while effective themselves, can be formulated and administered in the form of their pharmaceutically acceptable addition salts for purposes of stability, convenience of crystallization, increased solubility, and the like.

Preferred pharmaceutically acceptable addition salts include salts of mineral acids, for example, hydrochloric acid, sulfuric acid, nitric acid, and the like; salts of monobasic carboxylic acids, such as, for example, acetic acid, propionic acid, and the like; salts of dibasic carboxylic acids, such as, maleic acid, fumaric acid, oxalic acid, and the like; and salts of tribasic carboxylic acids, such as, carboxysuccinic acid, citric acid, and the like.

The xanthines can be administered orally, for example, with an inert diluent or with an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the compounds can be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums, and the like. These preparations should contain at least 0.5% of active compound, but the amount can be varied depending upon the particular form and can conveniently be between 4.0% to about 70% of the weight of the unit. The amount of xanthine in such compositions is such that a suitable dosage will be obtained. Preferred compositions and preparations according to the present invention are prepared so that an oral dosage unit form contains between about 1.0 mgs and about 300 mgs of active compound.

Tablets, pills, capsules, troches, and the like can contain the following ingredients: a binder, such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient, such as starch or lactose; a disintegrating agent, such as alginic acid, Primogel, corn starch, and the like; a lubricant, such as magnesium stearate or Sterotes; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; or flavoring agent, such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier, such as a fatty oil.

Other dosage unit forms can contain other materials that modify the physical form of the dosage unit, for example, as coatings. Thus, tablets or pills can be coated with sugar, shellac, or other enteric coating agents. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and preservatives, dyes, colorings, and flavors. Materials used in preparing these

compositions should be pharmaceutically pure and non-toxic in the amounts used.

For purposes of parenteral therapeutic administration, the xanthines can be incorporated into a solution or suspension. These preparations should contain at least 0.1% of the aforesaid compound, but may be varied between 0.5% and about 50% of the weight thereof. The amount of active compound in such compositions is such that a suitable dosage will be obtained. Preferred compositions and preparations according to the present invention are prepared so that a parenteral dosage unit contains between 0.5 mg to 100 mgs of the active compound.

Solutions or suspensions of the xanthines can also include the following components: a sterile diluent, such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents, such as benzyl alcohol or methyl parabens; antioxidants, such as ascorbic acid or sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid; buffers, such as acetates, citrates or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

While dosage values will vary with the specific disease condition to be alleviated, good results are achieved when the xanthines of formula (I) are administered to a subject requiring such treatment as an effective oral, parenteral or intravenous dose or from 0.1 to 25 mg/kg of body weight per day. A particularly preferred effective amount is about 1.0 mg/kg of body weight per day. In general, daily dosages will vary from 10–1,000 mg, preferably 100–600 mg per day.

It is to be understood, however, that for any particular subject, specific dosage regimens should be adjusted to the individual need and the professional judgment of the person administering or supervising the administration of the xanthines. It is to be further understood that the dosages set forth herein are exemplary only and that they do not, to any extent, limit the scope or practice of the invention.

This invention will now be described in greater detail in the following Examples.

EXAMPLES

To demonstrate the effectiveness of the claimed invention, a compound of the general formula I was tested to demonstrate inhibition of the activity of both *in vitro*-generated human IL-1 and other leukocyte derived cytokines, and purified human IL-1. Though a variety of compounds within the general formula (I) are effective in inhibiting the activities of IL-1 and other leukocyte derived cytokines, they will be exemplified with regard to 1,3dibutyl 7-(2-oxopropyl)xanthine (DBPOX) as a particularly preferred form of the invention.

Materials:

The compound 1,3-dibutyl 7-(2-oxopropyl)xanthine (DBOPX) was prepared according to the procedures described in U.S. Pat. No. 4,242,345. Interleukin-1: Purified human monocyte IL-1(IL-1β), and diluent were purchased from Cistron Biotechnology, Pine Brook, N.J. The human IL-1 used in these experiments was purified human monocyte interleukin-1. The diluent was PBS-0.1% bovine serum albumin (diluent). IL-1 contained $<$50pg/μg LPS by limulus amebocyte lysate assay. One LAF unit of IL-1 activity is defined as the amount of IL-1 which causes half-maximal incorporation of 3H-thymidine by murine [C^3H]thymocytes in the presence of concanavalin A [0.5 μg/ml].

Recombinant human tumor necrosis factor (alpha; rh-TNF): The rh-TNF was purchased from Genzyme Corp, (Boston, MA). It was produced in *E. coli* and was purified by phenyl sepharose chromatography and FPLC to a final purity of greater than 99% as determined by analysis on SDS acrylamide gels stained with both Coomassie Brilliant Blue R250 and silver staining. It has a molecular weight of 36,000 daltons by gel filtration on Superose 12 (FPLC) and consists of 2 dimers of 17,000 daltons each. It was supplied sterile in phosphate-buffered saline containing 0.1% bovine serum albumin as a carrier protein (data supplied by Genzyme). Just before use, the rh-TNF was diluted in Hanks balanced salt solution containing 0.1% human serum albumin.

The other materials were purchased as follows: Dimethyl sulfoxide (DMSO), n-formyl methionyl leucyl phenylalanine (FMLP; 10mM stock solution in DMSO was stored in 20 μl aliquots at $-70°$ C.), heparin, cytochrome c type VI from horse heart, and superoxide dismutase from bovine liver (SOD; stock solutions at 5 mg/ml in Hanks balanced salt solution were stored in 100 μl aliquots at 70° C.) (Sigma Chemical, St. Louis, Mo.); Neutrophil isolation medium (NIM: Los Alamos Diagnostics, Inc., Los Alamos, N.M.); Hanks balanced salt solution (HBSS), Minimum essential medium (MEM) and Medium 199 (M199) (Whittaker, M. A. Bioproducts, Walkersville, Md.); Dulbecco's phosphate buffered saline (PBS; GIBCO Laboratories, Grand Island, N.Y.); Limulus Amebocyte Lysate Test (LAL; Associates of Cape Cod, Inc., Woods Hole, Ma.); scrubbed nylon fiber (3 denier type 200) (Fenwal Laboratories, Deerfield, Ill.); Litex and Agarose type HSA (Accurate Chemical and Scientific Corp., Hicksville, N.Y.).

PMN preparation: Purified PMN (98% PMN and $<$95% viable by trypan blue exclusion) containing $<$1 platelet per 5 PMN and $<$50pg/ml LPS (LAL assay) were obtained from normal heparinized (10 Units/ml) venous blood by a one-step ficoll-hypaque separation procedure (NIM). The PMN were washed 3 times with HBSS or MEM. Residual RBC were lysed by hypotonic lysis for the PMN oxidative burst assays.

Mononuclear leukocyte conditioned medium: Mononuclear leukocyte conditioned media was prepared by incubating washed mixed mononuclear leukocytes (3×10^6/ml) from NIM separation in medium 199 (M199) containing 10% fresh autologous serum for 18 hrs. at 37° C. (10% CO_2) with or without LPS (5ng/ml) in Lab-Tek Flaskettes (Miles Inc., Naperville, Ill.) The suspension was centrifuged 150g$\times$10 min., and then the superratant was filtered (0.45 micron pore) and frozen ($-70°$ C.).

Statistics: The results are reported as the mean $\pm$ SEM. Pvalues were determined by using a 2-tailed student t-test.

EXAMPLE 1

Cell Chemotaxis

Chemotaxis under agarose was quantitated by the method of Nelson et al., J. Immunol., 115, 1650–1656 (1975). Purified PMN (5×10^6 PMN) were incubated for 15 min. at 37° C. in a total volume of (40ul, 60 μl, 90 μl) HBSS with or without DBOPX (as specified) and then

were incubated for 30 min. more at 37° C. in a total volume of 0.1 ml with or without LPS (0.2ng/40 μl), LPS stimulated mononuclear leukocyte conditioned medium (40 μl), IL-1 (15 units/60 ul) diluent (60 ul) or rh-TNF (100 units/10 μl). The migration to FMLP (100nM) was measured after 2 hrs. incubation at 37° C.

DBOPX increased chemotaxis inhibited by IL-1, TNF, or LPS stimulated mononuclear leukocyte conditioned medium as shown in FIGS. 1, 2 and 3.

EXAMPLE 2

PMN Adherence To Nylon

PMN adherence was determined by a modified method of MacGregor. Purified PMN were incubated in 0.1 ml medium 199 with or without DBOPX (as specified) containing LPS, or LPS stimulated mononuclear leukocyte conditioned medium for 30 min. at 37° C. After incubation HBSS (0.9 ml) and autologous serum (10 μl) were added to the cell suspensions. The cell suspensions were applied to the top of prewarmed (37° C.) 60mg nylon columns packed to the 0.3 ml mark on a plastic 1 ml syringe. The columns were allowed to elute for 30 min. at 37° C. and the number of PMN in both the pre- and post-column samples counted. The results are expressed as percent PMN adherence to the nylon.

DBOPX (10 μg/ml) diminished PMN adherence to nylon augmented by LPS stimulated mononuclear leukocyte conditioned medium as shown in FIG. 4.

EXAMPLE 3

PMN Oxidative Burst

Cytochrome c reduction: Purified PMN (2 to 4×10^6) were suspended in a total volume of 80 ul HBSS with or without DBPOX (as specified) and were incubated for 15 min. at 37° C. with or without SOD (200 units/sample). IL-1 (5 Units/20 μl), LPS (0.1 μg/20 ul), LPS stimulated mononuclear leukocyte conditioned medium (20 μl), or IL-1 diluent were then added and the cells incubated for 30 min. more at 37° C.

HBSS (0.4 ml) and cytochrome c (50 μl; final concentration 120 μM) were added to all samples. FMLP (100μM) was added. The samples were incubated for 10 min. more at 37° C. then iced, and centrifuged ($2000\times g$ for 10 min.). The optical density of the supernatants was read at a wavelength of 550 nm and the nmoles of SOD-inhibitable superoxide/10^6 PMN calculated using the extinction coefficient of 2.11×10^4 cm^2/mmole (reduced-oxidized).

DBOPX (0.1–100 μg/ml) decreased PMN superoxide production when the PMN had been primed with IL-1, TNF, and stimulated with FMLP as is evident from FIG. 5. DBOPX decreased PMN superoxide production when the PMN had been primed with LPS stimulated mononuclear leukocyte conditioned medium as shown in FIG. 6.

EXAMPLE 4

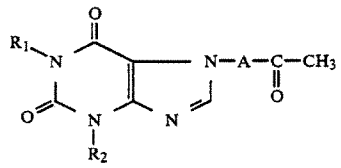
PMN Deqranulation (Release of Lvsozyme)

PMN (4×10^6) were suspended in HBSS (0.08 ml) with or without DBOPX (as specified) and incubated for 15 min. (37° C.). Then LPS (0.1 ng/0.02 ml) or LPS stimulated mononuclear leukoycte conditioned medium (0.02 ml) was added to the samples and incubated 30 min. more. HBSS (0.9 ml) and FMLP (10 ul; $10-^7$M final concentration) was added to all samples. The samples were incubated for 10 min. and then iced and centrifuged ($2000\times g$ for 10 min.). The supernatants were poured off and the lysozyme content determined by measurement of changes in the optical density of a suspension of *Micrococcus lysodeikticus* after addition of the supernatants using the method described in *J. Bacteriol.*, 58:731–736 (1949). DBOPX decreased the release of lysozyme from PMN primed with LPS stimulated mononuclear leukocyte conditioned medium and then stimulated with FMLP as is evident from FIG. 7.

Numerous modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described herein.

What is claimed is:

1. A method of treating a human to inhibit tissue injury accompanying inflammation resulting from leukocyte activity induced by cytokines produced in response to an inflammatory stimulus in the human, wherein the method comprises administering to said human at least one 7(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

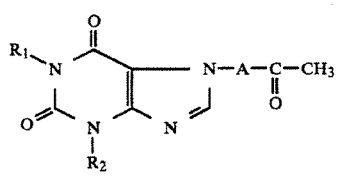
R_1 and R_2 are the same or different and are selected from the group consisting of straight chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to inhibit activity of human interleukin-1, human tumor necrosis factor, or the activity of other human leukocyte-derived human cytokines on polymorphonuclear leukocytes or monocytes in said human to thereby inhibit said tissue injury.

2. A method of claim **1**, wherein said xanthine is 1,3 dibutyl 7-(2-oxo-propyl)xanthine.

3. A method of treating a human to inhibit tissue injury accompanying inflammation resulting from leukocyte activity induced by cytokines produced in response to an inflammatory stimulus in the human, wherein the method comprises administering to said human at least one 7(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

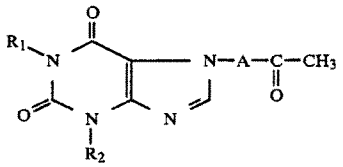
R_1 and R_2 are the same or different and are selected from the group consisting of straight chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to modulate the inflammatory effect of human interleukin-1, human tumor necrosis factor, or other human leukocyte-derived cytokines on polymorphonuclear leukocytes or monocytes by counteracting the inhibitory effect on cell movement in said human.

4. A method of claim **3**, wherein said xanthine is 1,3dibutyl 7-(2-oxo-propyl)xanthine.

5. A method of treating a human to inhibit tissue injury accompanying inflammation resulting from leukocyte activity induced by cytokines produced in response to an inflammatory stimulus in the human, wherein the method comprises administering to said human at least one 7(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

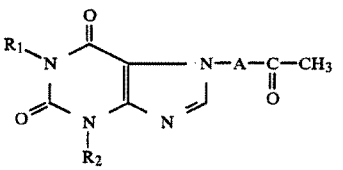
R_1 and R_2 are the same or different and are selected from the group consisting of straight chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to inhibit the stimulatory effect of human interleukin-1, human tumor necrosis factor, or other human leukocyte-derived cytokines or adherence of polymorphonuclear leukocytes or monocytes in said human to thereby inhibit said tissue injury.

6. A method of claim **5**, wherein said xanthine is 2,3dibutyl 7-(2-oxo-propyl)xanthine.

7. A method of treating a human to inhibit tissue injury accompanying inflammation resulting from leukocyte activity induced by cytokines produced in response to an inflammatory stimulus in the human, wherein the method comprises administering to said human at least one 7-(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

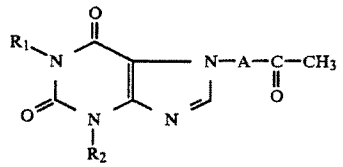
R_1 and R_2 are the same or different and are selected from the group consisting of straight chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to inhibit stimulatory effect of human interleukin-1, human tumor necrosis factor, or other human leukocyte-derived human cytokines n oxidative burst of stimulated polymorphonuclear leukocytes in said human to thereby inhibit said tissue injury.

8. A method of claim **7**, wherein said xanthine is 1,3dibutyl 7-(2-oxo-propyl)xanthine.

9. A method of treating a human to inhibit tissue injury accompanying inflammation resulting from leukocyte activity induced by cytokines produced in response to an inflammatory stimulus in the human, wherein the method comprises administering to said human at least one 7(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

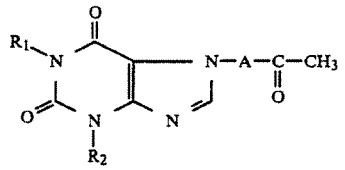
R_1 and R_2 are the same or different and are selected from the group consisting of straight chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to inhibit the activity of human interleukin-1, human tumor necrosis factor, or other human leukocyte-derived human cytokine on degranulation of stimulated polymorphonuclear leukocytes in said human to thereby inhibit said tissue injury.

10. A method of claim **9**, wherein said xanthine is 1,3dibutyl 7-(2-oxo-propyl)xanthine.

11. A method of treating a human to inhibit tissue injury accompanying inflammation resulting from leukocyte activity induced by cytokines produced in response to an inflammatory stimulus in the human, wherein the method comprises administering to said human at least one 7(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

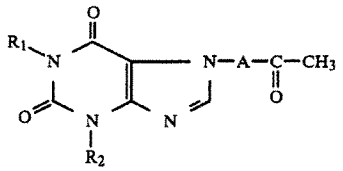
R_1 and R_2 are the same or different and are selected from the group consisting of straight chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to inhibit the effect of human interleukin-1 or human tumor necrosis factor on oxidative burst or degranulation of stimulated neutrophils in said human to thereby inhibit said tissue injury.

12. A method of claim **11**, wherein said xanthine is 1,3-dibutyl 7-(2-oxo-propyl)xanthine.

13. A method of treating a human to alleviate inflammatory pathological effects of sepsis, septic shock, endotoxic shock, gram-negative sepsis, toxic shock syndrome, the adult respiratory syndrome, and the fever and cachexia of AIDS, wherein the method comprises administering to said human at least one 7-(oxoalkyl) 1,3-dialkyl xanthine of the formula



in which

R_1 and R_2 are the same or different and are selected from the group consisting of straight-chain or branched chain alkyl radicals with 2 to 6 carbon atoms, cyclohexyl, straight-chain or branched chain alkoxyalkyl and hydroxyalkyl radicals; and

A represents a hydrocarbon radical with up to 4 carbon atoms which can be substituted by a methyl group;

wherein said xanthine is administered to said human in an amount sufficient to inhibit activity of human interleukin-1, human tumor necrosis factor, or the activity of other human leukocyte-derived human cytokines on polymorphonuclear leukocytes or monocytes in said human to thereby inhibit said effects.

14. A method of claim **13**, wherein said xanthine is 1,3-dibutyl 7-(2-oxo-propyl)xanthine.

* * * * *